Remarks

I. Summary of the Office Action

In the Office Action dated November 17, 2005 (hereinafter "the Office Action"), claims 220-272, 275-332 and 335-465 are pending. The Examiner has withdrawn from consideration claims 230-237, 245-247, 250-257, 259-267, 277-332, 335-354, 372-379, 387-389, 409-411 and 419-458, as being drawn to nonelected species. *See* Office Action at page 2.

At pages 2-3 of the Office Action the Examiner has rejected claims 220-229, 238-244, 248, 249, 258, 355-371, 380-386, 390-408 and 459-465 under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for the self-antigen species VEGFR2, IL5, IL13, and eotaxin, allegedly does not reasonably provide enablement for the full scope of self antigens.

At pages 3-4 of the Office Action the Examiner has provisionally rejected claims 220-229, 238-242, 244, 248, 249, 258, 355-371, 380-384, 386, 390-408, 412-418 and 459-465 under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 1, 2, 5-8, 12-20, 22, 25-32, 35-40, 42-45, 65-67, 69, 74-87, 89 and 95-120 of copending U.S. Application No. 10/289,454.

At page 4 of the Office Action the Examiner has objected to claims 268-272, 275, 276 and 412-418 as being dependent upon a rejected base claim.

In view of the following remarks, Applicants respectfully traverse the Examiner's objections and rejections. Applicants therefore respectfully request that the Examiner reconsider and withdraw the objections and rejections, and allow the presently pending claims.

II. Withdrawn Claims

The Examiner has withdrawn from consideration claims 230-237, 245-247, 250-257, 259-267, 277-332, 335-354, 372-379, 387-389, 409-411 and 419-458 as being drawn to nonelected species. Office Action at page 2. In view of the following remarks, Applicants respectfully believe that the generic or linking claims are allowable, and therefore respectfully request the rejoining, examination and allowance of these additional claims encompassing species not selected for initial search and examination, in accordance with 37 C.F.R. § 1.141.

III. Objections to the Claims

At page 4 of the Office Action the Examiner has objected to claims 268-272, 275, 276 and 412-418 as being dependent upon a rejected base claim, but would be otherwise allowable if rewritten in independent form. Applicants thank the Examiner for identification of otherwise allowable subject matter. However, for at least the reasons detailed below, Applicants respectfully believe that the rejected base claims are now allowable and respectfully request that the Examiner reconsider and withdraw the objection, and allow claims 268-272, 275, 276 and 412-418.

IV. Rejections Under 35 U.S.C. § 112, First Paragraph

The Examiner has rejected claims 220-229, 238-244, 248, 249, 258, 355-371, 380-386, 390-408 and 459-465 under 35 U.S.C. § 112, first paragraph, as allegedly exceeding the scope enabled by the specification, especially with regard to the genus of self antigens. In making this rejection, the Examiner has asserted that:

> [T]he specification, while being enabling for the self-antigen species VEGFR2, IL5, IL13, and eotaxin (as allowed in co-pending application 10/289454), does not reasonably provide enablement for the full scope of self antigens. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims. The generic claims embrace a multitude of species of self antigens. The only disclosed use for all of the species is the prevention or treatment of diseases or disorders where the self product is suspected of involvement. The lists of diseases and disorders to be treated with self antigens include a very broad range of different conditions, and a number of conditions where prevention or treatment is notoriously difficult, such as systemic lupus erythematosus (SLE), rheumatoid arthritis, multiple sclerosis, and Alzheimer's disease. The list also includes diabetes, where an immune response against the self-antigen would be reasonably expected to exacerbate the autoimmune disease rather than treat it. Therefore, one skilled in the art would have reason to doubt assertions that all of the species embraced in the genus could be used in prevention or treatment without additional guidance. Considering the broad scope of the claims, the limited teachings in the specification, and the absence of working examples of prevention or treatment of diseases or disorders for many of the species recited in the claims, it is concluded that undue experimentation would be required to use the full scope of the claimed products in the manner suggested in the specification.

Office Action at pages 2-3. Applicants respectfully disagree with these assertions and traverse the rejection.

(a) The Examiner has not met the burden of establishing non-enablement

The enablement requirement of 35 USC § 112, first paragraph, is satisfied if the claimed invention is enabled so that any person skilled in the art can make and use the invention without undue experimentation. *See In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). In order to establish a *prima facie* case of non-enablement the Examiner has the initial burden to set forth a reasonable basis to question the enablement provided for the claimed invention. *See In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). To satisfy this burden, "it is incumbent

upon the Patent Office . . . to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." *See In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971) (emphasis in original); *see also In re Wright*, 999 F.2d 1557, 1562 (Fed. Cir. 1993) ("Without a reason to doubt the truth of the statements made in the patent application, the application must be considered enabling.").

The Examiner has acknowledged at page 2 of the Office Action that the "specification [is] enabling for the self-antigen species VEGFR2, IL5, IL13, and eotaxin." Applicants assert that the enablement of these species demonstrates that one of ordinary skill in the art would consider that the full genus of self antigens is enabled by the specification, allowing such a person to practice the claimed invention. Accordingly, the scope of the claims is within the enablement provided by the specification.

Despite such evidence the Examiner asserts that the "specification does not reasonably provide enablement for the full scope of self antigens." Office Action at page 2. However, the Examiner provides no evidence why one of ordinary skill in the art would not consider that the specification enables the full scope of the claims. Applicant therefore respectfully asserts that the Examiner's burden to prove nonenablement has not been met, as the Examiner has not provided relevant proof that one of ordinary skill in the art would consider that the claims are not enabled. Rather, the rejection is based on the application of an unreasonable standard that is not found in the law, such that the objective enablement of numerous species within the scope of the claims is still not considered as reasonable evidence of enablement of the broad scope of the claims (*see*

(b), below); and/or requiring that the Applicants prove the enablement of therapy according to a heightened standard (*see* (c), below). Therefore, Applicants respectfully assert that the Examiner has not met the burden of establishing non-enablement and therefore the present specification must be taken as enabling the presently claimed invention.

(b) Sufficient embodiments are enabled to enable the full scope of the claims

To the extent that the present rejection relies on the Examiner's assertion that "the absence of working examples of prevention or treatment of diseases or disorders for many of the species recited in the claims" (Office Action at page 3), this reliance is misplaced. First, Applicants are not required to demonstrate enablement of all species within the claim. The Federal Circuit has recently reiterated that a *single* operative embodiment within the claim may enable a generic claim as "the enablement requirement is met if the description enables *any* mode of making and using the invention." *Invitrogen Corp. v. Clontech Laboratories, Inc.*, 429 F.3d 1052, 1071 (Fed. Cir. 2005), citing *Johns Hopkins University v. Cellpro, Inc.*, 152 F.3d 1342, 1361 (Fed. Cir. 1998) (emphasis added). Noting that an operable embodiment within the claim had been fully described, the court further stated that:

> Enablement does not require the inventor to foresee every means of implementing an invention at pains of losing his patent franchise. Were it otherwise, claimed inventions would not include improved modes of practicing those inventions. Such narrow patent rights would rapidly become worthless as new modes of practicing the invention developed, and the inventor would lose the benefit of the patent bargain.

Invitrogen, 429 F.3d at 1071. Accordingly, the Examiner's acknowledgement of four working embodiments within the claim suffices to enable the entire genus. Indeed,

Applicants are not even required to disclose a *single* working example. "Nothing more than objective enablement is required, and therefore it is irrelevant whether this teaching is provided through broad terminology or illustrative examples." *In re Wright*, 27 U.S.P.Q.2d 1510, 1561 (Fed. Cir. 1999); *see also In re Borkowski*, 422 F.2d 904, 908 (C.C.P.A. 1970) ("a specification need not contain a working example if the invention is otherwise disclosed in such a manner that one skilled in the art will be able to practice it without an undue amount of experimentation."); *In re Long*, 151 U.S.P.Q. 640, 642 (C.C.P.A. 1966) ("absence of a working example does not in and of itself compel the conclusion that a specification does not satisfy the requirements of section 112."). Rather, the enablement requirement of 35 U.S.C. § 112, first paragraph, is satisfied if the claimed invention is enabled so that any person skilled in the art can make and use the invention without undue experimentation. *See In re Wands*, 858 F.2d 731, 737, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). For at least this additional reason, Applicants respectfully assert that the present claims are enabled.

(c) Enablement of the claims does not require demonstration of therapeutic efficacy.

One aspect of the Examiner's rejection is the allegation that:

> the only disclosed use for all of the species is the prevention or treatment of diseases or disorders. . . . [o]ne skilled in the art would have reason to doubt assertions that all of the species embraced in the genus could be used in prevention or treatment [of a broad range of diseases and disorders] without additional guidance."

Office Action at pages 2-3. Applicants respectfully disagree, and also assert that this aspect of the rejection is an incorrect application of the law to the present claims.

Under U.S. patent law, to be entitled to a patent on an invention an inventor must enable the public to make and use the invention. *See, e.g., In re Wands*, 858 F.2d 731, 737, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). Applicants are merely required to enable that which is claimed. Claims 220-262, 265-272, 275-280, 283-285, 292-297, 300-306, 309-316, 319-320, 329-332, 335-355, 357, 362-459 and 461 are directed to compositions, claims 359-361 and 463-465 are directed to a process of producing a composition, and claims 356, 358, 460, 462 are directed of methods of inducing an immune response in an animal. Accordingly, if one of ordinary skill in the art can make or use the invention for the induction of an immune response, the presently claimed invention is enabled.

Applicants also respectfully disagree with the assertion that "the only disclosed use for all of the species is the prevention or treatment of diseases or disorders." Office Action at page 2. The presently claimed invention is also useful for at least the production of an immune response against self antigens, and can therefore be used for the production of antibodies against self antigens. This is a specific and credible utility within 35 U.S.C. § 101. Therefore, the present claims have both utility and are enabled for that utility.

Furthermore, even if some level of therapeutic efficacy *were* to be required to enable the claimed invention, Applicants would *not* have to demonstrate treatment of any and all diseases associated with self antigens. Applicants would merely have to demonstrate that the scope of the claims was commensurate with that enabled by the specification. *Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 750 F.2d 1569, 1577, 224 USPQ 409, 414 (Fed. Cir. 1984). The Examiner has accepted that at least four

therapeutic embodiments are enabled. For reasons detailed above, Applicants respectfully assert that this is evidence that the genus is enabled.

In making the rejection, the Examiner has stated that "the list [of self antigens] also includes diabetes, where an immune response against the self-antigen would be reasonably expected to exacerbate the autoimmune disease rather than treat it." Office Action at page 3. As an allegedly nonworking embodiment would not destroy the enablement of the claims, this is an incorrect application of law. *Atlas Powder,* 750 F.2d 1569. Moreover, Applicants also assert that it is an incorrect application of fact. The specification provides for the production of an immune response against, for example, lymphotoxin-β (paragraph [0396]), which has been specifically associated with diabetes. *See, e.g.,* Wu *et al.* Reversal of spontaneous autoimmune insulitis in nonobese diabetic mice by soluble lymphotoxin receptor, *J. Exp. Med.* 193: 1327-32 (2001) (reporting that lymphotoxin receptor-Ig chimeric protein prevented and reversed the development of diabetes in NOD mice; cited as AT73 in the first supplemental IDS); Levisetti *et al.,* Absence of lymph nodes in NOD mice treated with lymphotoxin-beta receptor immunoglobulin protects from diabetes, *Diabetes 53*(12):3115-9 (2004). Both documents are attached herewith as Exhibits A and B. Accordingly, a person of ordinary skill in the art would reasonably consider that the production of an immune response against lymphotoxin-β would be useful in the prevention and even treatment of diabetes. For at least these reasons, Applicants respectfully assert that this aspect of the rejection is traversed and should be withdrawn.

(d) The specification also enables additional embodiments within the scope of the claims

Not only does the present application provide sufficient disclosure to enable those of ordinary skill in the art to make and use the claimed invention, it *also* specifically demonstrates the reduction to practice of numerous *compositions* comprising a virus-like particle with a self-antigen. Furthermore, it *also* demonstrates the production of an *immune response* against self antigens following the immunization of an animal with a composition comprising a virus-like particle associated with at least the following self-antigens: VEGFR2 (Examples 12 and 49); Angiotensin (Example 42); and TNFα (Example 53). Thus, not only is the "specification enabling for the self-antigen species VEGFR2, IL5, IL13, and eotaxin," as acknowleged by the Examiner (Office Action at page 3), but the specification *also* discloses additional "working examples" of a composition comprising other self antigens, methods of making such compositions, and immune responses to such self antigens following immunization. Hence, even if working embodiments were required for the specification to enable the presently claimed invention (which they do not, under *Wright, Borkowski*, and *Long*), the present specification clearly provides working examples of a broad range of self antigens. Hence, in the absence of proof otherwise, Applicants assert that the present specification clearly meets and, indeed, exceeds, the enablement requirements.

For at least these additional reasons, Applicants assert that the rejection should be withdrawn.

(e) The specification enables therapeutic embodiments

Even if the claims were read to require that the specification enable therapeutic embodiments, Applicants assert that this heightened standard is also met. Applicants remind the Examiner that they are not required to demonstrate therapeutic efficacy to enable claims to therapy. There is no requirement for clinical data to prove that an application is in compliance with 35 U.S.C. § 112, first paragraph. *See Cross v. Iizuka*, 753 F.2d 1040, 1050 (Fed. Cir. 1985); *see In re Brana*, 51 F.3d 1560, 1567-68 (Fed. Cir. 1995) (holding that animal testing results are sufficient to establish whether one skilled in the art would believe that a pharmaceutical compound has an asserted clinical utility for the purposes of compliance with the enablement requirement of 35 U.S.C. § 112, first paragraph).

The specification describes a number of self antigens and their association with disease states. One of ordinary skill in the art would consider that the production of antagonists to these self antigens, such as antibodies, would be effective in treatment or prevention of such disease states. *See, e.g.*, paragraph [0296]. The specification provides for compositions that effectively induce immune responses against antigens, including against self antigens. Having established the nexus between a self antigen and a disease state, all that would be required would be to demonstrate the induction of an immune response for one of ordinary skill to consider that the specification enables claims to therapy aimed at the disease state. *See, e.g.*, paragraph [0015]. The specification further provides that:

> The invention provides vaccine compositions suitable for use in methods for preventing and/or attenuating diseases or conditions which are caused or exacerbated by "self" gene products (*e.g.*, tumor necrosis factors). Thus, vaccine compositions of the invention include compositions which

> lead to the production of antibodies that prevent and/or attenuate diseases or conditions caused or exacerbated by "self" gene products.

Specification at paragraph [0287]. Accordingly, the specification teaches that the compositions of the present invention *are* suitable for use in methods for preventing or treating diseases or conditions which are caused by self antigens.

More specifically, the specification provides for a detailed description of examples of self antigens and related diseases thereof. These include VEGFR2 (e.g. paragraphs [0292]-[0296], Examples 11, 12, 48, 49 and 50), IL-5 (e.g. paragraphs [0354]-[0360], Example 10), IL-13 (e.g. paragraphs [0350]-[0353], Example 9), and eotaxin (e.g. paragraphs [0382]-[0385]). The Examiner has already accepted that these embodiments are enabled. However, the specification also provides additional exemplary embodiments.

The specification at Example 6 and paragraphs [0324]-[0333] describes compositions of antigens derived from ***RANKL*** coupled to VLPs and that these compositions can be used for the treatment of osteoporosis (*see* paragraph [0328]).

Paragraphs [0401]-[0407] and Examples 52 and 53 describe antigens derived from ***TNF-α*** and the synthesis of compositions comprising these antigens coupled to VLPs. It is well known that TNF-α plays an important role in autoimmune diseases such as rheumatoid arthritis (*see, e.g.,* Klareskog *et al.*, Rheumatoid arthritis and its animal models: the role of TNF-α and the possible absence of specific immune reactions, *Curr. Opin. Immunol. 11*:657–662 (1999); and Myers *et al.*, Collagen-induced arthritis, an animal model of autoimmunity, *Life Sciences 61*(19):1861-1878 (1997), both of which are provided herewith as Exhibits C and D).

Many further examples of self antigens and related diseases thereof are described in great detail throughout the specification, including in paragraphs [0292]-[0299] and [0323]-[0423]. Given the explicit teachings of the specification and the knowledge available to those of ordinary skill in the art, the present claims must be considered fully enabled.

Furthermore, even if one of ordinary skill in the art had not been so taught by the present specification, it would not require undue experimentation to determine that the claimed compositions are useful for preventing or treating diseases or conditions which are caused by self antigens. Hence the present specification fully enables the presently claimed invention.

(f) Post-filing data demonstrate that the specification enables the presently claimed invention

Even if the Examiner had met the burden of establishing a *prima facie* case of non-enablement, Applicants may rebut such an allegation by providing post-filing evidence that, by practicing the teachings of the specification, one of ordinary skill in the art can make and use the claimed invention without undue experimentation. *See Brana,* 51 F.3d at 1567 ("[A post-filing declaration] does not render an insufficient disclosure enabling, but instead goes to prove that the disclosure was in fact enabling when filed."). Applicants provide herewith a Declaration of Martin Bachmann Under 37 C.F.R. § 1.132 ("the Declaration"), which describes numerous examples of post-filing art that demonstrates that the specification, at the time of filing, enabled the full scope of the claims.

The Declaration demonstrates that the specification of the present application provides for compositions of VLPs to which self antigens, or peptides or fragments thereof, are conjugated, methods of making such compositions, the use of such compositions as immunogens and, further, for therapeutic (including treatment and prevention) purposes. These self antigens include TNFα, RANKL, angiotensin, and ghrelin. Dr. Bachmann summarizes in Section 10 of the Declaration that:

> Therefore, in accordance with the disclosure of the present application, we have demonstrated that numerous compositions which comprise self antigens, or peptides or fragments thereof, conjugated to virus-like particles are able to overcome the natural tolerance of the immune system toward self proteins, to induce an immune response against the self antigens in immunized animals, resulting in antibodies against the self antigens, and that such immune responses are effective in preventing and/or treating diseases associated with such self antigens in recognized animal models of such diseases.

Accordingly, such post-filing disclosures demonstrate that the present application enables the practice of the invention with a wide array of embodiments and demonstrates the enablement of the full scope of the claims.

(g) Summary

For at least the reasons outlined above, the full scope of the pending claims is enabled by the specification. Reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, first paragraph, is therefore respectfully requested.

V. Obviousness-Type Double Patenting

At pages 3-4 of the Office Action the Examiner has provisionally rejected claims 220-229, 238-242, 244, 248, 249, 258, 355-371, 380-384, 386, 390-408, 412-418 and 459-465 under the judicially created doctrine of obviousness-type double patenting as

allegedly being unpatentable over claims 1, 2, 5-8, 12-20, 22, 25-32, 35-40, 42-45, 65-67, 69, 74-87, 89 and 95-120 of copending U.S. Application No. 10/289,454. Applicants respectfully traverse the rejection.

However, solely to advance prosecution and not in acquiescence to this rejection, Applicants provide herewith a Terminal Disclaimer under 37 C.F.R. § 1.32(c) over the term of the patent issuing from U.S. Application No. 10/289,454. Applicants respectfully request consideration and acceptance of this Terminal Disclaimer, and the reconsideration and withdrawal of the provisional rejection of the present claims for obviousness-type double patenting over U.S. Application No. 10/289,454.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully requests that the Examiner reconsider and withdraw all presently outstanding objections and rejections. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Brief Definitive Report

Reversal of Spontaneous Autoimmune Insulitis in Nonobese Diabetic Mice by Soluble Lymphotoxin Receptor

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Abstract

One striking feature of spontaneous autoimmune diabetes is the prototypic formation of lymphoid follicular structures within the pancreas. Lymphotoxin (LT) has been shown to play an important role in the formation of lymphoid follicles in the spleen. To explore the potential role of LT-mediated microenvironment in the pathogenesis of insulin-dependent diabetes mellitus (IDDM), an LTβ receptor–immunoglobulin fusion protein (LTβR–Ig) was administered to nonobese diabetic mice. Early treatment with LTβR–Ig prevented insulitis and IDDM, suggesting that LT plays a critical role in the insulitis development. LTβR–Ig treatment at a late stage of the disease also dramatically reversed insulitis and prevented diabetes. Moreover, LTβR–Ig treatment prevented the development of IDDM by diabetogenic T cells in an adoptive transfer model. Thus, LTβR–Ig can disassemble the well established lymphoid microenvironment in the islets, which is required for the development and progression of IDDM.

Key words: adhesion molecule • autoimmune diabetes • insulitis • lymphotoxin • lymphotoxin β receptor

Introduction

Insulin-dependent diabetes mellitus (IDDM) is a T cell–mediated autoimmune disease in which the insulin-secreting β cells are selectively destroyed after weeks or months of insulitis. Much of our current knowledge about the complex pathogenesis of IDDM derives from the studies of nonobese diabetic (NOD) mice (1–5). In NOD mice, infiltration of autoreactive T cells into the islets is essential for the development of IDDM. Studies performed in these animals have revealed that the influx of T cells into the pancreas is associated with an increased expression of adhesion molecules. However, factors that upregulate adhesion molecules in the pancreas have not been identified. Lymph node–like structures with de novo formation of lymphoid follicles are gradually established within the islets over 2–3 mo, with initial cellular infiltration starting at 3–4 wk of age (1, 3, 4, 6, 7). While the formation of lymphoid follicular structures is a prototypic feature of chronic progressive inflammation (6–8), the molecular mechanisms by which the lymphoid follicles are formed and the role of these follicles in the pathogenesis of IDDM have not been well defined.

The interaction between membrane lymphotoxin (LT) and its receptor is essential for the development and maintenance of the lymphoid microenvironment (9–15). $LT\alpha^{-/-}$ mice and wild-type mice treated with LTβ receptor–immunoglobulin fusion protein (LTβR–Ig) exhibit altered lymphoid microenvironment. This effect is likely caused by a failure to induce lymphoid tissue chemokines and adhesion molecules (12–15). These results imply that LT may be a master cytokine responsible for the formation of lymphoid structures in chronic inflammation and autoimmune diseases, such as IDDM. Here, we report that administration of LTβR–Ig reverses the formation of lymphoid follicles, prevents β cell destruction by autoreactive T cells, and forestalls the development of IDDM.

Materials and Methods

Reagents and Mice. The generation and production of recombinant LTβR–Ig has been described previously (15, 16). The murine LTβR–human Ig in culture supernatants of BHK/VP16 (15) or Chinese hamster ovary cells (16) was purified on a protein A column. No difference could be found between the two preparations. Human Ig was obtained from Biogen, Inc. or Sigma-Aldrich. Female NOD mice were purchased from The Jackson Laboratory and maintained under specific pathogen–free conditions at the University of Chicago. Antibodies to vascular cell adhesion molecule (VCAM), peripheral lymph node addressin

Q. Wu and B. Salomon contributed equally to this study. Drs. Bluestone and Fu should both be considered senior authors of this article.

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(PNAd; MECA 79), mucosal addressin cell adhesion molecule 1 (MAdCAM-1; MECA367), B220, CD4 (GK1.5), CD8 (3.155), and CD11c (N418) were purchased from PharMingen.

LTβR–Ig Treatment and Measurement of Blood Glucose. NOD mice were given 100 μg of LTβR–Ig or human Ig intraperitoneally once per week for 3 wk or as indicated. The glucose concentration in blood obtained from a tail vein was measured using SureStep® strips (Johnson and Johnson). Diabetes was monitored by levels of blood glucose. Animals were considered diabetic after two consecutive measurements of ≥250 mg/dl of glucose.

Adoptive Transfer. Recipient NOD mice (7–9 wk) were irradiated with 6 Gy (199 rads/min) using a ^{137}Cs irradiator. 2 h later, the mice were injected intravenously with 2×10^7 splenocytes from diabetic NOD mice in 0.2 ml of PBS, followed by intraperitoneal injection with 100 μg of LTβR–Ig, anti–MAdCAM-1, or control human Ig.

Histology and Immunohistochemistry. Pancreatic tissue was collected 10 d after the last LTβR–Ig treatment. Hematoxylin and eosin staining was performed on 6-μm sections of 10% formalin-fixed tissue. Infiltration into islets was examined microscopically and counted by a third party pathologist. Sections were prepared on multiple levels, and 20–40 randomly chosen islets per mouse were semiquantitatively classified according to the severity of insulitis: moderate and severe insulitis are defined to be less or more than half of the structure was infiltrated, respectively. Additionally, some of the pancreata and spleen were frozen at −70°C in OCT compound for immunohistology. 6-μm cryostat sections were incubated with rat antibodies to CD3 (L363–29B), VCAM, PNAd (MECA 79), MAdCAM-1 (MECA367), B220, CD4 (GK1.5), CD8 (3.155), hamster antibody to CD11c (N418), or species- and isotype-specific nonreactive control mAbs overnight at 4°C. The next day, room temperature incubation with biotinylated rabbit anti–rat or goat anti–hamster secondary antibody (Vector Laboratories) for 30 min was followed by a 1-h incubation with streptavidin–horseradish peroxidase complex (Vector Laboratories).

Results

Prevention of Diabetes by the Administration of LTβR–Ig. Typically, 65–75% of untreated NOD mice in our colony develop IDDM by 25 wk. To study the role of membrane LT at an early stage of the disease, 3–4-wk-old NOD female mice were treated weekly with LTβR–Ig (100 μg/wk) for 3 wk. Only one of the NOD mice treated with LTβR–Ig developed IDDM by 32 wk, while 67% of the NOD mice treated with control Ig developed IDDM by 26 wk (Fig. 1 A). To study whether LTβR–Ig treatment reduced IDDM after the initial phase, female NOD mice were treated with the soluble receptor at 6–7 wk of age, a time when many islets are infiltrated with autoreactive T cells. Treatment with LTβR–Ig at this time similarly prevented the development of IDDM, while the majority of control mice developed the disease by 25 wk (Fig. 1 B). These results suggest that LT is essential for the early development of IDDM.

Previous studies have shown that $CD4^+CD25^+$ regulatory cells control spontaneous autoimmune diabetes, even at late stages, by limiting the destructive infiltrate in the islets (17). We previously reported that both $B7^{-/-}$ mice and $CD28^{-/-}$ NOD mice exhibited severe insulitis and accelerated disease as a consequence of a reduced number of these suppressor cells (17, 18). To study whether LT was

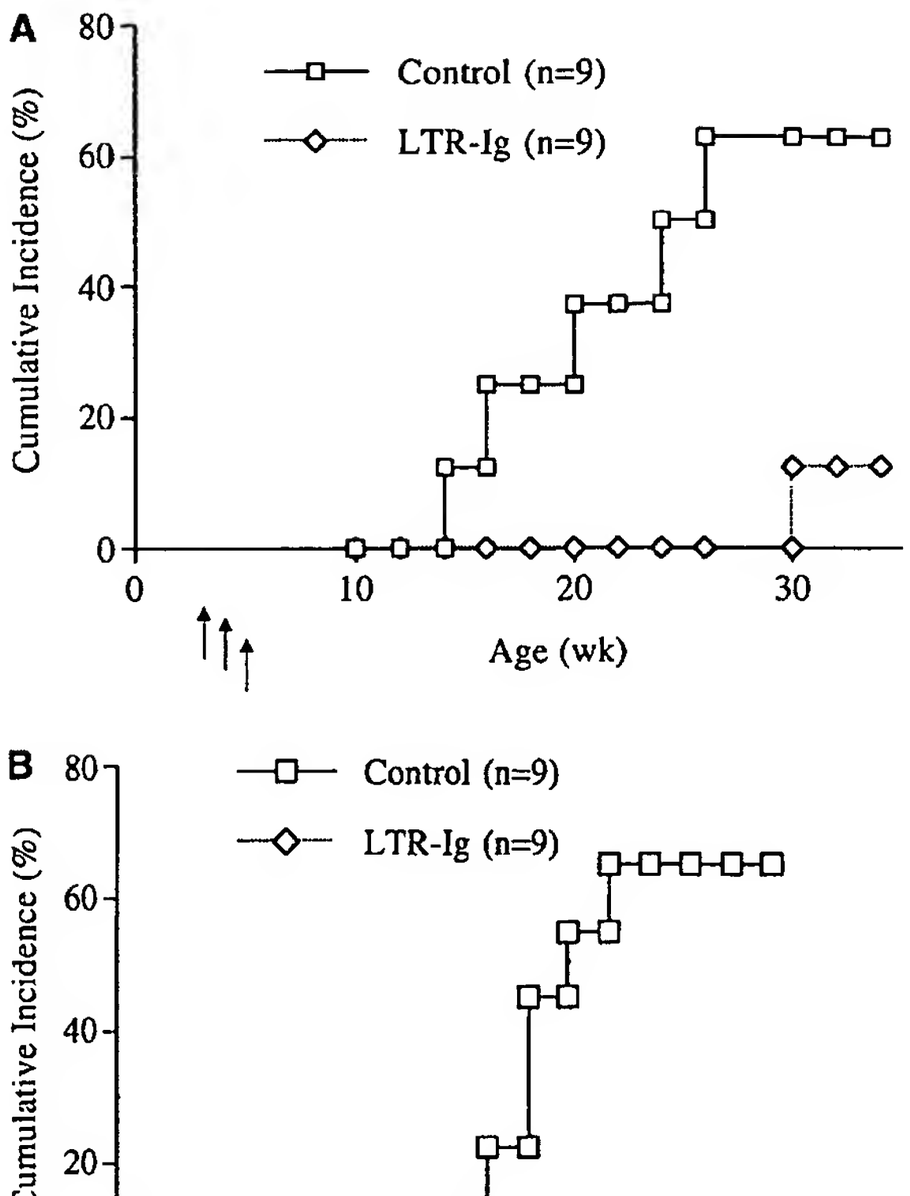

Figure 1. LTβR–Ig treatment prevents diabetes in young NOD mice. Young NOD mice (n = 10) 3–4 (A) and 6–7 wk of age (B), were treated intraperitoneally with LTβR–Ig or control human Ig for 3 wk. (C) Young $CD28^{-/-}$ NOD mice (n = 5 per group) of 3 wk of age were treated intraperitoneally with LTβR–Ig or PBS for 4 wk. Incidence of diabetes was evaluated at weekly intervals.

functioning by altering this regulatory T cell subset, $CD28^{-/-}$ NOD mice, deficient in $CD4^+CD25^+$ T cells, were treated weekly with LTβR–Ig or control Ig for 4 wk starting at 3 wk of age and examined for disease incidence. All of the control mice developed IDDM by 11 wk of age, similar to the time course observed in previous experiments. In contrast, there was a delay in disease onset in the LTβR–Ig-treated mice. In fact, some of the mice were free of IDDM for an additional 10–13 wk (Fig. 1 C). Similar retarded diabetes was found in $CD28^{-/-}$ NOD mice that were only treated twice with the fusion protein beginning at the ages of 4 and 5 wk. However, the number of $CD4^+CD25^+$ T cells in the spleens of LTβR–Ig-treated mice remained comparable with that in the control group (data not shown). Therefore, these results suggest a critical role for LT in the development of IDDM in this accelerated model (Fig. 1 C), which is independent of CD28 and cannot be attributed to the $CD4^+CD25^+$ T cell pathway.

Reversal of Islet Destruction by Preexisting Diabetic T Cells Using LTβR–Ig. By 10 wk, most islets in NOD mice show severe infiltration by autoreactive T cells, accompanied by early signs of islet cell destruction. Very few reagents have been shown to block the development of IDDM at this late stage (5, 19). To determine whether the effect of LTβR–Ig could prevent the development of IDDM at this late phase, 10-wk-old NOD mice were similarly treated weekly with LTβR–Ig over 3 wk. None of the LTβR–Ig-treated NOD mice became diabetic, whereas most of control NOD mice developed IDDM by 25 wk (Fig. 2 A). To study whether LTβR–Ig treatment just retarded the development of IDDM for a few more days or had a prolonged protection, we extended our observation up to 38 wk and found that none of the LTβR–Ig-treated NOD mice became diabetic. More astonishingly, two doses of LTβR–Ig blocked the development of diabetes in 85% of prediabetic NOD mice (11/13) treated as late as 14 wk of age, a time point when some NOD mice (two mice from each group) were already diabetic, with nearly all the islets attacked by autoreactive T cells (Fig. 2 B). Only 15% (2/13) of prediabetic mice treated with LTβR–Ig developed IDDM at 20 wk of age, whereas most of the control Ig–treated group (63%) developed IDDM by 18 wk of age. These findings suggest that LT also plays an important role in the late phases of the disease. However, treatment failed to reverse IDDM in the mice that were already diabetic at the time of treatment.

The transfer of splenocytes from diabetic NOD mice into irradiated NOD recipient results in diabetes within a few weeks, due to acute infiltration of donor autoreactive T cells into islets. To further address whether the administration of LTβR–Ig can prevent diabetogenic T cells from destroying islet cells, splenocytes from diabetic NOD mice were transferred into irradiated NOD recipients treated with a single dose of LTβR–Ig. The development of IDDM in these irradiated mice was prevented (Fig. 2 C). These results support a model wherein the use of LTβR–Ig can prevent the development of IDDM mediated by preexisting autoreactive T cells in the circulation.

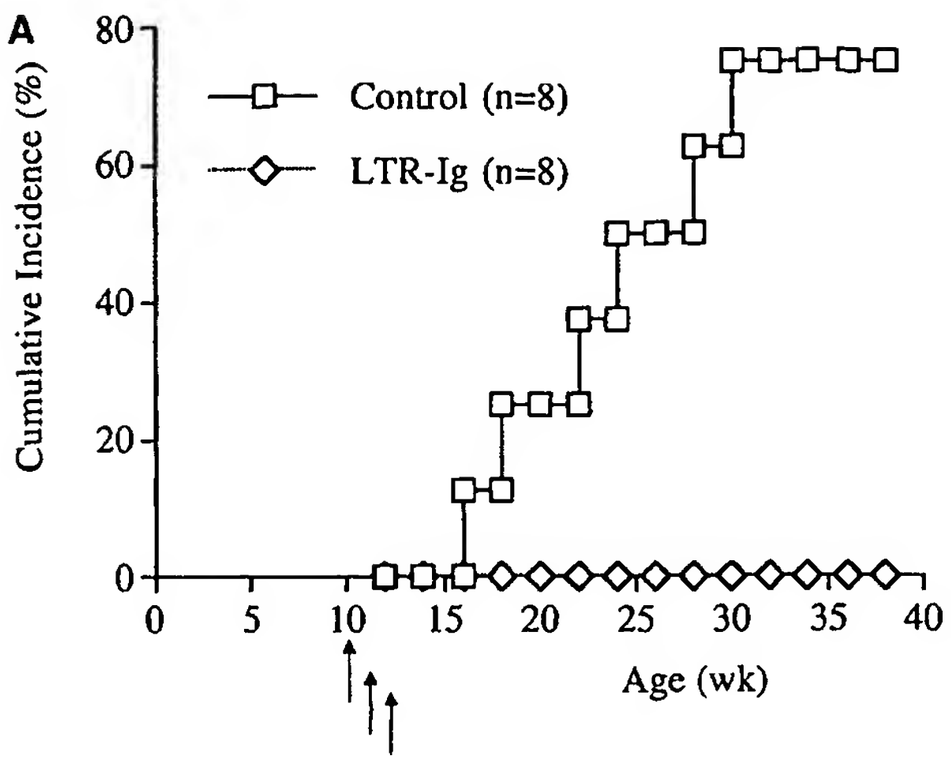

Figure 2. LTβR–Ig treatment blocks autoreactive T cell–mediated islet destruction. (A) NOD (10 wk of age) female mice (n = 10) were given weekly intraperitoneal injection of 100 μg of LTβR–Ig or human Ig for 3 wk. (B) NOD (14 wk of age) female mice were given weekly intraperitoneal injection of 100 μg of LTβR–Ig (n = 13) or human Ig (n = 8) for only 2 wk. Blood glucose levels were checked weekly after the end of treatment (A and B). (C) Sublethally irradiated NOD female mice (10 mice per group at 7–9 wk of age) were injected intravenously with 2×10^7 splenocytes from mice with diabetes and treated with a single intraperitoneal injection of 100 μg of LTβR–Ig or human Ig. Blood glucose levels were checked weekly starting 1 wk after adoptive transfer.

Prevention and Resolution of Insulitis after LTβR–Ig Therapy. One striking feature of IDDM in NOD mice is the prototypic formation of lymphoid follicular structures within the pancreas. The profound blockade of different stages of IDDM by LTβR–Ig treatment may be attributed to the reduction or even reversal of LT-mediated lymphoid structures in the pancreas. To test this, 3–4- or 10-wk-old NOD mice were treated weekly with LTβR–Ig and control Ig for 3 wk. 10 d after the end of treatment, the pancreata were collected for hematoxylin and eosin staining. As seen in Fig. 3, the number with destructive insulitis and its severity was greatly reduced in both treated groups of mice during the inductive phase (Fig. 3 A). The percentage of islets in an early treated group of mice displaying overall infiltration with formation of lymphoid structures (28.4%) was considerably lower than that observed in the control mice (70.1%; Fig. 3 B). In NOD mice treated at the late stage (10 wk), only 15% of islets developed insulitis, and 3% of those showed the formation of lymphoid structure inside β cells by 14 wk. In contrast, 82% of the corresponding control group demonstrated insulitis and 69% exhibited severe insulitis (Fig. 3 B). The data also demonstrated that the number and scope of infiltrating cells in LTβR–Ig-treated mice at the age of 14 wk was reduced far below the level of untreated mice at the age of 8–9 wk (before the treatment). These results indicate that LTβR–Ig treatment not only prevents but also resolves the formation of lymphoid structures in the pancreas. The data also reveal that the infiltration of inflammatory cells and the development of lymphoid follicles may be a dynamic process and can be reversed once the influx of cells is blocked.

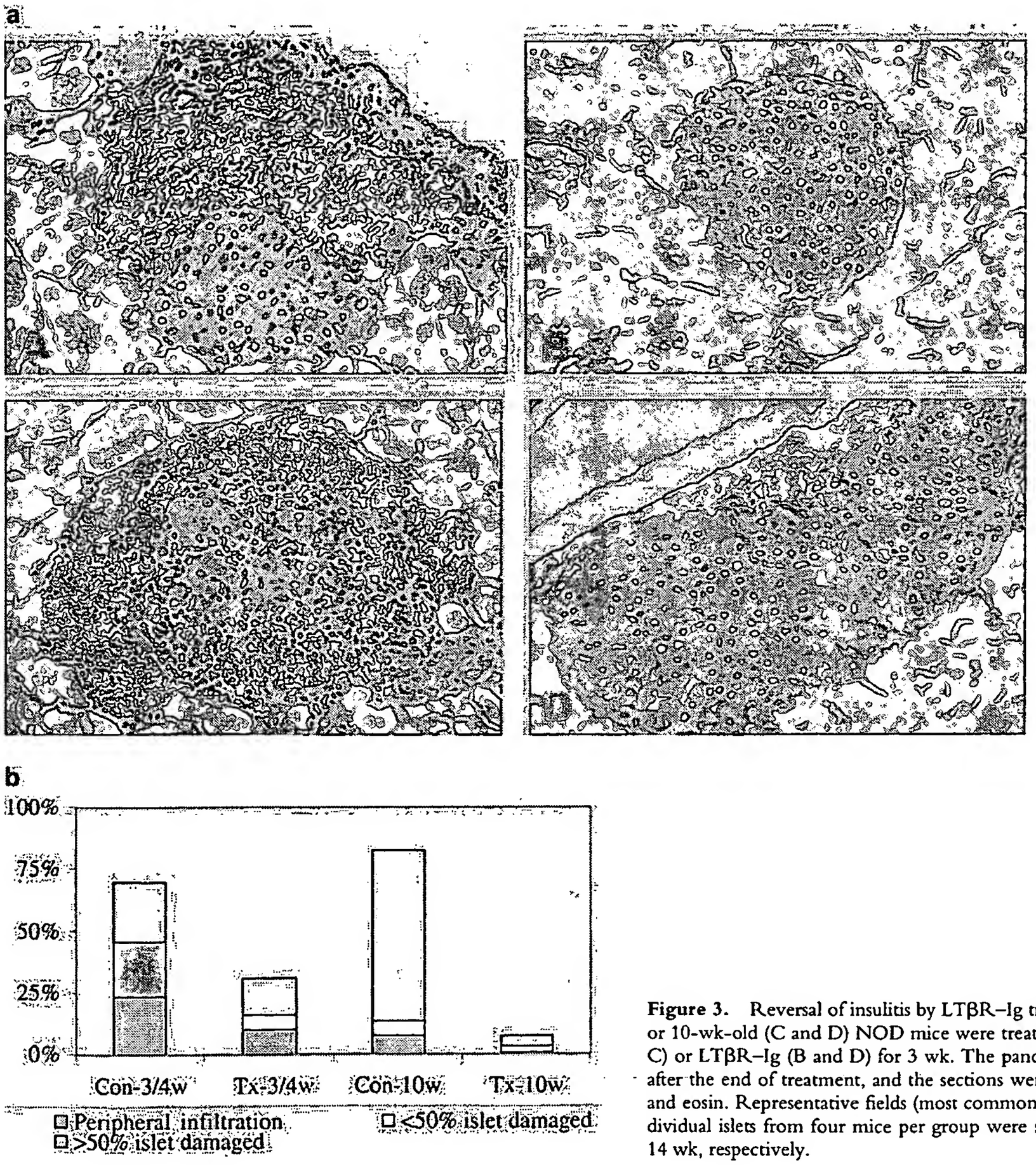

Figure 3. Reversal of insulitis by LTβR–Ig treatment. (a) 4- (A and B) or 10-wk-old (C and D) NOD mice were treated with control Ig (A and C) or LTβR–Ig (B and D) for 3 wk. The pancreata were collected 10 d after the end of treatment, and the sections were stained by hematoxylin and eosin. Representative fields (most common fields) are shown. (b) Individual islets from four mice per group were scored for insulitis at 8 or 14 wk, respectively.

Discussion

Our study demonstrates that the treatment of NOD mice with LTβR–Ig can be effective at blocking both the initiation and effector phases of diabetes. More importantly, such treatment reverses insulitis and prevents the formation of lymphoid follicles, even when insulitis is well established and diabetogenic T cells are clearly present. Such treatment at either early or late stage has prolonged the protection, suggesting that short-term treatment with LTβR–Ig could significantly alter the course of IDDM. Several possible mechanisms may be associated with this treatment. First, as both LT and LIGHT bind LTβR in vitro, these effects may arise from blockage of LIGHT signaling. LIGHT is a costimulatory molecule through its receptor, herpes virus entry mediator, that has been shown to promote T cell proliferation and IFN-γ production (20). Its biological activity in vivo, however, has not been well defined. Further dissection of the role of LIGHT is difficult due to the lack of reagents that effectively block LIGHT activity in vivo. Anti-LTβ antibody has an extremely short half-life of 1 d, while the half-life of LTβR–Ig is 7 d (16). Second, LT may be a critical cytokine for the development of the $CD4^+CD25^+$ T cells that regulate autoimmunity. Although LTβR–Ig inhibited the development of the disease in the $CD4^+CD25^+$ cell–reduced CD28 knockout NOD mice, there was no increase in the number of regulatory T cells in the spleen during or after treatment. There was also no increase of $CD4^+CD25^+$ cells in wild-type NOD mice after treatment. Thus, it appears unlikely that the effects of LTβR–Ig treatment are mediated through $CD4^+CD25^+$ T cells.

The most likely mechanism by which LTβR–Ig prevents the development of IDDM is through LT-mediated expression of adhesion molecules and chemokines and the formation of lymphoid tissues (9–15). Compared with other adhesion molecules, such as VCAM-1 and PNAd, the expression of MAdCAM-1 is more closely associated with the development of insulitis. In fact, treatment of NOD mice with anti–MAdCAM-1 antibody (MECA367) alone can reduce insulitis and prevent the development of IDDM (21). Interestingly, both LTβR–Ig and anti-LTβ antibody can very efficiently block the expression of MAdCAM-1 in the spleen (12, 13). We found that the expression of MAdCAM-1 in the pancreas and spleen was readily reduced after the administration of LTβR–Ig. To directly test whether blocking MAdCAM-1 can prevent the migration of autoreactive T cells, NOD mice that received T cells from diabetic mice were treated with anti–MAdCAM-1 (MECA367) antibody. Such treatment could prevent or retard the development of IDDM (data not shown). Together, these data suggest that LT-mediated adhesion molecules, such as MAdCAM-1, are at least partially responsible for the development of IDDM. It is possible that the expression of MAdCAM-1 and other adhesion molecules is required for the formation of insulitis or lymphoid follicles in the pancreas. It has been proposed recently that the expression of membrane LT is required for the formation of large lymphoid follicles in the islets of B lymphocyte chemokine–mediated transgenic mice (8). We have previously showed that the migration of dendritic cells into the spleen is impaired in LTβR–Ig-treated mice (15). It is possible that the chemokines and adhesion molecules reduced by LTβR–Ig treatment slow the influx of inflammatory cells into the pancreas. In this study, we demonstrated that LTβR–Ig reduced the formation of lymphoid follicles and even reversed severe insulitis in NOD mice. Taken together, these data suggest that LT-mediated microenvironment is essential for the development of lymphoid structure that is required for the development of IDDM.

The generation of a genetic defect of the LT gene in NOD mice is another potential model that could be used to address the role of LT in the development of IDDM. Several disadvantages to this model exist, however. First, intercrossing of the NOD mice with $LT\alpha^{-/-}$ mice to retain a defect in both LT and the MHC loci is an impractical task, as the locus of LT is very close to that of MHC class II, which is a key genetic locus for the development of IDDM. Second, $LT\alpha^{-/-}$ mice lack organized lymph nodes, which may be critical for the development of IDDM by itself. Such compound defects may complicate the interpretation of the results. On the other hand, the use of soluble receptor has several advantages. We can correlate different phases of the disease with the timing of administration of fusion protein. Long-term impact after the termination of treatment can be readily assessed.

Resolution of severe insulitis by LTβR–Ig treatment suggests that the migration of inflammatory cells into the islets is a dynamic process in which these cells may constantly move in and out of the target tissue. Interference with this dynamic process may reduce inflammation, which prevents further activation of autoreactive T cells and tissue damage. Interestingly, alteration of the LT-mediated microenvironment by LTβR–Ig is a transient effect that ends once the treatment is terminated. For example, the expression of splenic MAdCAM-1 and the development of B cell follicles was gradually restored 4–5 wk after the termination of LTβR–Ig treatment. Thus, the observation that short-term LTβR–Ig treatment has a profound and prolonged impact on islet destruction may provide a new avenue for the study of pathogenesis and for the treatment of this disease or other autoimmune diseases. In the future, it will be interesting to explore whether LTβR–Ig treatment may prevent islet cell rejection by autoreactive or alloreactive T cells.

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Absence of Lymph Nodes in NOD Mice Treated With Lymphotoxin-β Receptor Immunoglobulin Protects From Diabetes

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Pregnant nonobese diabetic (NOD) mice were treated with lymphotoxin-β receptor immunoglobulin fusion protein (LTβR-Ig) or control human immunoglobulin on days embryonic day 11 (E11) and E14, and offspring were followed for the development of anti–β-cell antibodies, islet pathology, and hyperglycemia. The development of anti–β-cell surface antibodies was abrogated in treated mice compared with controls. Autopsy examination of the mice at 30 weeks of age revealed normal development of secondary lymphoid structures in the control animals; however, mice treated with LTβR-Ig had no axillary, inguinal, popliteal, or peripancreatic lymph nodes. Histological examination of the pancreata of the control mice revealed a severe and destructive mononuclear cellular infiltrate in the islets, whereas the islets of the LTβR-Ig–treated mice were devoid of any insulitis. None of the LTβR-Ig–treated mice (n = 22) developed diabetes; in contrast, 80% of the control mice (n = 46) developed diabetes at 1 year of age. The LTβR-Ig–treated mice did not contain diabetogenic T-cells. However, the treated mice developed diabetes upon inoculation with diabetogenic T-cells. In this model of spontaneous autoimmune diabetes, secondary lymphoid structures, most likely the peripancreatic lymph nodes, were essential for the development of pathologic anti–β-cell autoimmunity. *Diabetes* 53: 3115–3119, 2004

Recent studies have demonstrated an important role for local lymph nodes in the pathogenesis of tissue-specific autoimmune disease. For example, in a mouse model of autoimmune arthritis, the inhibition of lymph node development by in utero administration of lymphotoxin-β receptor-immunoglobulin fusion protein (LTβR-Ig) delayed and attenuated the course of disease (1). We chose to investigate the role of peripheral lymph nodes in the pathogenesis of autoimmune diabetes in the nonobese diabetic (NOD) mouse model of the disease using a similar approach. The peripancreatic lymph nodes accumulate diabetogenic T-cells and may be their major site of priming and activation (2–5). Moreover, surgical excision of the peripancreatic lymph nodes delayed and decreased the incidence of diabetes in NOD mice (6). The present study administering LTβR-Ig to pregnant NOD mice was initiated just at the time of the report on surgical excision of peripancreatic lymph nodes. Our studies in "nodeless" mice induced by LTβR-Ig confirm and extend these results.

The essential role of the lymphotoxin (LT)β signaling pathway in lymphoid organogenesis was revealed by the analysis of mutant mice in which LTβ signaling did not take place: LTβR (LTβ receptor)-deficient, LTα-deficient, and LTβ-deficient mice failed to develop lymph nodes (7–11). Moreover, the LTβ system also plays a crucial role in the establishment and maintenance of the organized lymphoid structures that are present in tissue-specific autoimmune disease. The therapeutic effects of LTβR-Ig have been studied in rodent models of arthritis, experimental autoimmune encephalomyelitis, colitis, uveitis, and autoimmune diabetes (12–16). In a report from Bluestone and Fu's (16) laboratories, administration of the LTβR-Ig inhibited the development of diabetes and actually reversed active inflammation. Diabetogenic T-cells transferred into nondiabetic mice together with the fusion protein failed to induce diabetes. In entirety, their results indicated that the development of the lymphocytic lesion that develops in the NOD mouse required LTβ signaling. Results similar to these were obtained from McDevitt's group (17) but using transgenic mice that expressed the LTβ fusion protein. Our studies differ from these two in that the mice were exposed to the soluble decoy receptors during gestation, disrupting lymph node development but leaving LTβ signaling intact in the adult mice (18).

RESEARCH DESIGN AND METHODS

NOD mice were originally obtained from The Taconic Laboratory and NOD.scid mice from The Jackson Laboratory (Bar Harbor, ME). Mice were followed for the development of diabetes by bimonthly blood glucose measurement, and diabetes was defined by values >250 mg/dl on two separate occasions. All mice were housed and cared for in accordance with the guidelines of the Washington University Committee for the Humane Care of Laboratory Animals and with the National Institutes of Health guidelines on laboratory animal welfare.

LTβR-Ig treatment. Timed pregnant NOD females were injected with 100 μg i.v. LTβR-Ig (gift of J. Browning, Biogen, by way of Drs. Laura Mandik-Nayak and Paul Allen) or control human immunoglobulin (Jackson ImmunoResearch) on embryonic day 11 (E11) and E14 as described (18).

Cell lines and antibodies. The NIT-1 cell line (19) was a gift from Dr. E.H. Leiter (The Jackson Laboratory). The monoclonal antibody SF1.1.1 was used to stain H-2K^d (Pharmingen, San Diego, CA).

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LTβR-Ig, lymphotoxin-β receptor immunoglobulin fusion protein.

A

	Control-Ig treated ($\times 10^6$)	LTβR-Ig treated ($\times 10^6$)
Total Splenocytes	92+/-21	102+/-27
CD4+	34	40
CD8+	14	15
B220+/CD19+	27	28
CD11c+	2	2
F4/80+	7	8

B

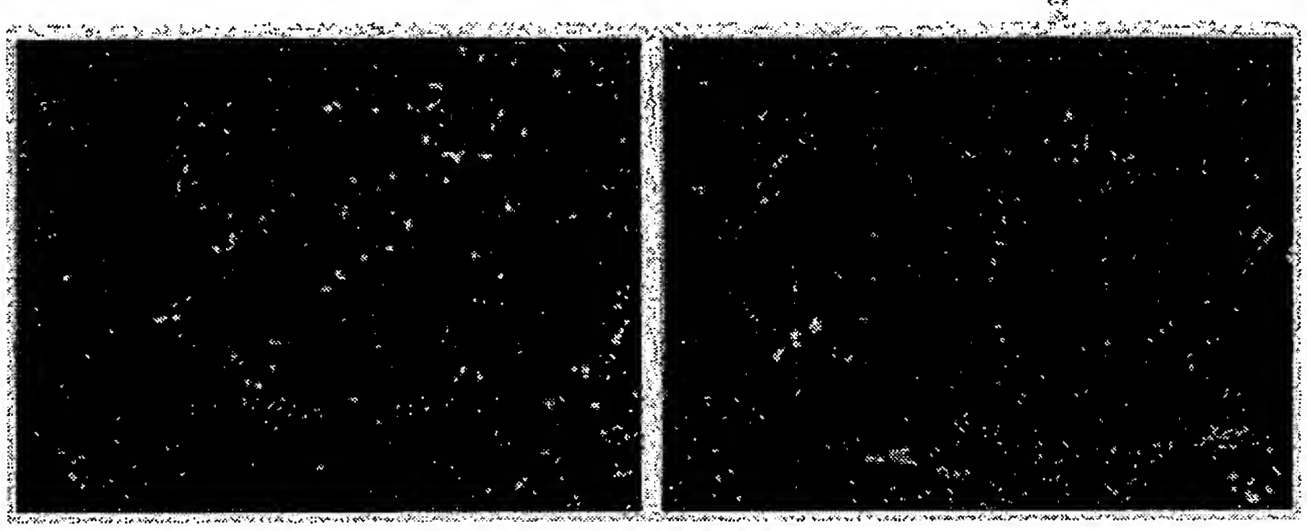

FIG. 1. LTβR-Ig–treated animals had normal splenic cellularity and architecture. The cellularity of the spleens from control immunoglobulin and LTβR-Ig mice were not different from one another (*A*). There was no statistically significant difference in the total number of cells or in the numbers of T-cells, B-cells, dendritic cells, and macrophages. The architecture of the spleens appeared normal in the nodeless mice. The white pulp contained a normal appearing periarteriolar lymphoid sheath (anti–CD3-FITC ([fluorescein isothiocyanate]: green) surrounded by normal appearing B-cells (anti–CD-19-PE: red) (*B*).

Fluorescence-activated cell sorter analysis of serum antibodies. NIT-1 cells were incubated first with mouse sera and then with a goat anti-mouse IgG-PE (Caltag, Burlingame, CA). Fluorescence-activated cell sorter (FACS) analysis was performed on a FACScan flow cytometer, and data analysis was performed with CellQuest software (Becton Dickinson, Mountain View, CA).
Histology and immunohistochemistry. Mice were killed by cervical dislocation, and pancreata were fixed in 10% formalin and then stained with hematoxylin and eosin. The spleens of control and nodeless animals were frozen and then stained with anti–CD3-FITC (fluorescein isothiocyanate) and anti–CD19-PE (Pharmingen).
Transfer into NOD.scid recipients. Splenocytes (2×10^7) from control and nodeless mice were transferred into NOD.scid recipients by intravenous tail vein injection. The mice were followed by blood glucose determination every 1–2 weeks.
Transfer of splenocytes into control and nodeless mice. Nodeless mice and 8-week-old NOD male mice were irradiated with 600 R and then received 3×10^7 splenocytes from diabetic NOD donors by intravenous tail injection.

RESULTS

LTβR-Ig–treated mice fail to develop lymph nodes. Mice from control immunoglobulin and LTβR-Ig–treated mothers were examined for the presence of lymph nodes. As previously reported, LTβR-Ig–treated mice were born without axillary, inguinal, and popliteal lymph nodes (18) and also lacked peripancreatic lymph nodes. The absence of peripancreatic lymph nodes was confirmed by en block resection and careful histological analysis of tissue containing the duodenum and head of the pancreas, including the superior mesenteric vessels. The spleens from the two groups of animals had normal cellularity and architecture (Fig. 1*A* and *B*). Specifically, there was no significant difference in the total number of cells or in the number of T-cells, B-cells, dendritic cells, and macrophages between the two groups of animals. The gross architecture of the spleens was preserved as demonstrated by normal appearing T- and B-cell zones.
LTβR-Ig treatment prevents diabetes. The offspring of LTβR-Ig–treated mice were followed for the development of hyperglycemia by weekly blood glucose measurement. Control mice developed diabetes with kinetics and a cumulative incidence equivalent to that found in our NOD mouse colony, with an incidence of 80% (36 of 46) at 1 year of age (Fig. 2). In contrast, none of the mice (0 of 22) of LTβR-Ig–treated mothers developed diabetes at 1 year. The pancreata of mice from both control and LTβR-Ig mice were examined histologically for the presence of inflammation and β-cell death. The control mice all developed a destructive inflammatory infiltrate, with marked loss of β-cell mass; in contrast, the islets from the LTβR-Ig–treated mice were devoid of infiltrate (Fig. 3). A weak peri-insulitis was found in the pancreas of some of the nodeless mice after 40 weeks of age; however, β-cell mass was preserved in these animals.
The development of anti–β-cell surface autoantibodies is attenuated by LTβR-Ig treatment. The NOD mouse spontaneously develops antibodies that bind to β-cell surface antigens between 4 and 8 weeks of age (20). To determine the role of lymph nodes in this process, control and experimental animals were examined for the presence of anti–β-cell autoantibodies at various time points. Serum antibody titers were assessed by flow cytometric analysis of NIT-1 cells stained with mouse serum and expressed as mean fluorescence intensity. At the time points examined (6, 10, and 30 weeks of age), the development of anti–β-cell antibodies was reduced by approximately half in the LTβR-Ig–treated group when compared with control animals ($P < 0.05$, Table 1). Of note, LTβR-Ig–treated mice did develop low titers of β-cell autoantibodies, as demonstrated by positive staining when compared with nonautoimmune strains of mice, such as B10.BR mice.
LTβR-Ig–treated mice do not harbor diabetogenic T-cells. To determine whether protected mice contained T-cells capable of inducing diabetes, splenocytes from LTβR-Ig–treated mice were transferred into NOD.scid

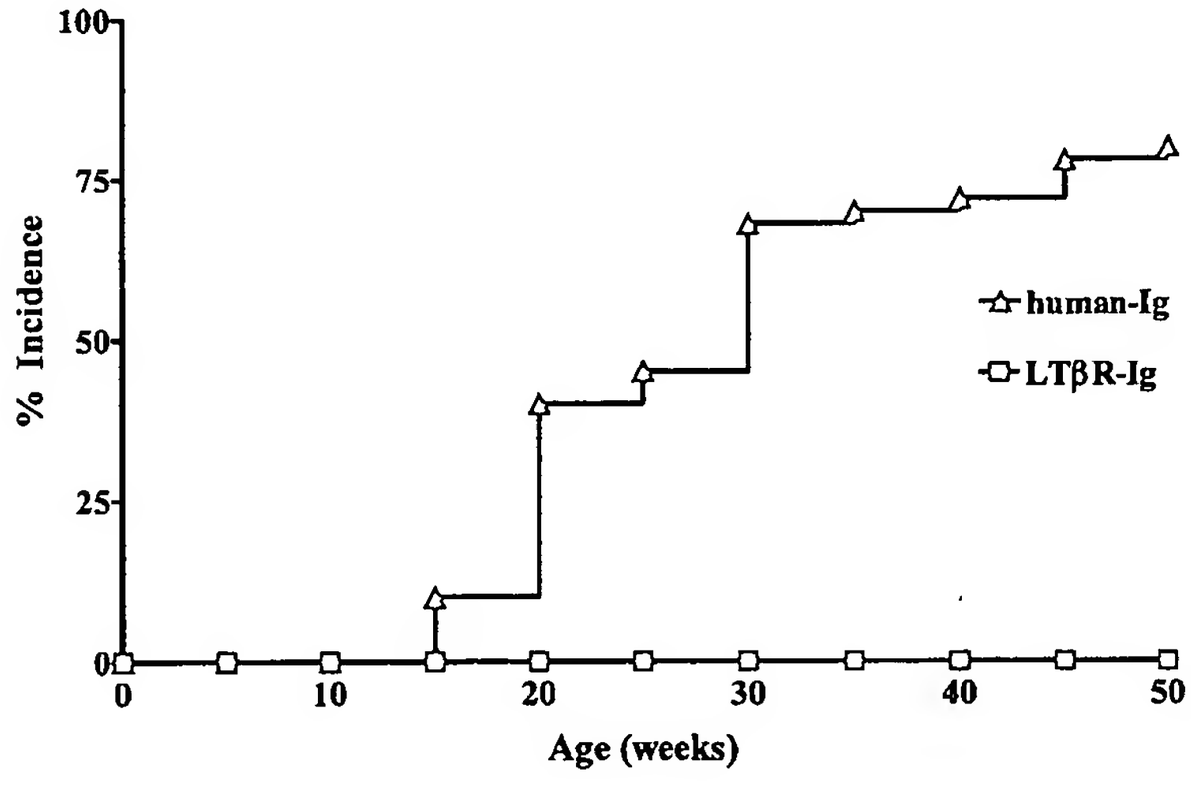

FIG. 2. Treatment with LTβR-Ig prevented diabetes. The cumulative incidence of diabetes in controls (△, $n = 46$) and LTβR-Ig–treated animals (□, $n = 22$) is presented above. At 1 year of age the incidence in control animals, males and females combined, was 80%, while none of the nodeless animals developed disease.

recipients. Splenocytes from LTβR-Ig–treated mice failed to transfer diabetes into NOD.scid recipients compared with controls. Splenocytes from diabetic control mice induced diabetes in all (13 of 13) recipients by 8 weeks posttransfer; however, splenocytes from nodeless mice induced diabetes in only 1 of 13 recipients at 25 weeks posttransfer (Fig. 4*A*). These results taken together indicate that the presence of secondary lymphoid organs plays a key role in the expansion and/or maintenance of a diabetogenic T-cell repertoire. However, the defect in T-cell development is not global, as demonstrated in experiments where NOD and nodeless mice were immunized with the protein antigen hen-egg white lysozyme intraperitoneally and the spleen T-cells were then tested for proliferation. Cells from both control and LTβR-Ig–treated mice proliferated to hen-egg white lysozyme, indicating that T-cells could respond in an antigen-specific manner to systemic immunization. Moreover, these findings are consistent with previously published data in similar model systems (13,14,21).

However, the ability of splenocytes from diabetic NOD mice to induce diabetes upon transfer into nodeless NOD mice was no different from controls (Fig. 4*B*). By 8 weeks posttransfer, 60% (3 of 5) had developed disease in the two groups, indicating that the peripancreatic lymph nodes are dispensable for the adoptive transfer of diabetes induced by primed T-cells.

DISCUSSION

This study, in conjunction with others (3,6,22), reveals the critical importance of secondary lymphoid structures, most likely the peripancreatic lymph nodes, in the pathogenesis of autoimmune diabetes in the NOD mouse. Mice without lymph nodes were completely protected from the development of diabetes and had reduced anti–β-cell autoantibodies and reduced numbers of pathogenic T-cells.

The complete protection from disease enjoyed by the nodeless mice is very likely the result of the interruption or prevention of a critical step in the disease process, namely the priming and expansion of β-cell–specific pathogenic T-cells, an event that requires the local concentration of diabetogenic antigens in the milieu of a lymph node. The failure of splenocytes from nodeless animals to transfer disease (1 of 13 at 25 weeks posttransfer) supports the conclusion that β-cell–reactive T-cells were not primed in the nodeless animals or that T-cells existed at a low frequency incapable of inducing disease when transferred into NOD.scid mice.

In contrast to the surgical removal of the peripancreatic lymph nodes at 3 weeks of age, which reduced the incidence of diabetes to ~20% at 30 weeks of age, (6) LTβR-Ig treatment provided complete protection from diabetes for over 1 year. It is possible that the critical priming event, presumably the encounter of β-cell–specific T-cells with antigen-presenting cells bearing relevant β-cell antigens, may occur before 21 days of age in a subset of animals. Interestingly, the nodeless mice produced anti–β-cell antibodies, although at half the titers compared with controls, while the mice treated with splenectomy had greatly reduced titers of anti-insulin antibodies. Taken together, these results clearly reveal the important role played by both the spleen and the peripancreatic lymph

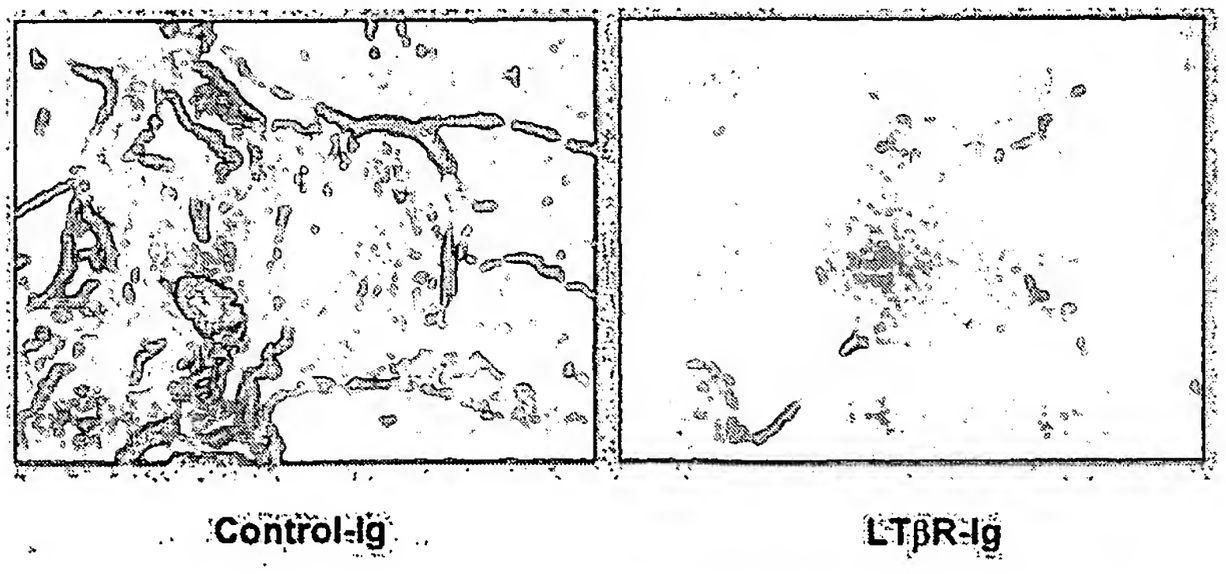

FIG. 3. Nodeless mice failed to develop insulitis. The islets of control mice developed a severe inflammatory infiltrate and marked loss of β-cell mass. The islets in the nodeless NOD mice were free of infiltrate, and β-cell mass was preserved. The histology shown above was taken from female animals at 30 weeks of age.

TABLE 1
Attenuation of anti–β-cell antibodies in LTβR-Ig–treated mice

	n	6 weeks old	10 weeks old	30 weeks old
Control immunoglobulin	21	18.4	42.3	30.4
LTβR-Ig	22	10.6	22.4	20.8

Data are average mean fluorescence intensity for each group of animals. The development of anti–β-cell surface antibody was attenuated in nodeless mice. The serum of nodeless NOD mice at 6, 10, and 30 weeks of age contains less anti–β-cell antibody when compared with controls ($P < 0.05$, paired t test). The serum of the nodeless mice was positive at 10 and 30 weeks when compared with B10.BR serum (mean fluorescence intensity 8.9, $n = 5$).

nodes in the production of a vigorous anti–β-cell humoral response.

Finally, as in the study of Gagnerault et al. (6), diabetes developed after the transfer of T-cells into the nodeless mice. This is an indication that the recognition by T-cells of diabetogenic antigens can take place in at least two sites, the local draining lymph node or the pancreas—the former is the favorable site for priming, the latter for the recruitment and development of already-activated T-cells. The peripancreatic node contains diabetogenic T-cells and is indispensable for T-cell induction, as the Gagnerault study and ours indicate (although the mice in our study have a complete absence of lymph nodes). In contrast, the islets can be infiltrated by diabetogenic T-cells independent of lymph nodes, indicating that primed T-cells do not require an intermediate migration through local nodes. The important question of whether the diabetogenic T-cell recognizes the antigen-presenting cells around the islets or within them needs to be determined.

Although the molecular identity of β-cell antigens relevant to disease pathogenesis have yet to be fully elucidated, this study, in addition to others, identifies the local lymph nodes as the critical site of T-cell priming. This knowledge of the anatomy of the disease process will hopefully facilitate the identification of disease relevant autoantigens.

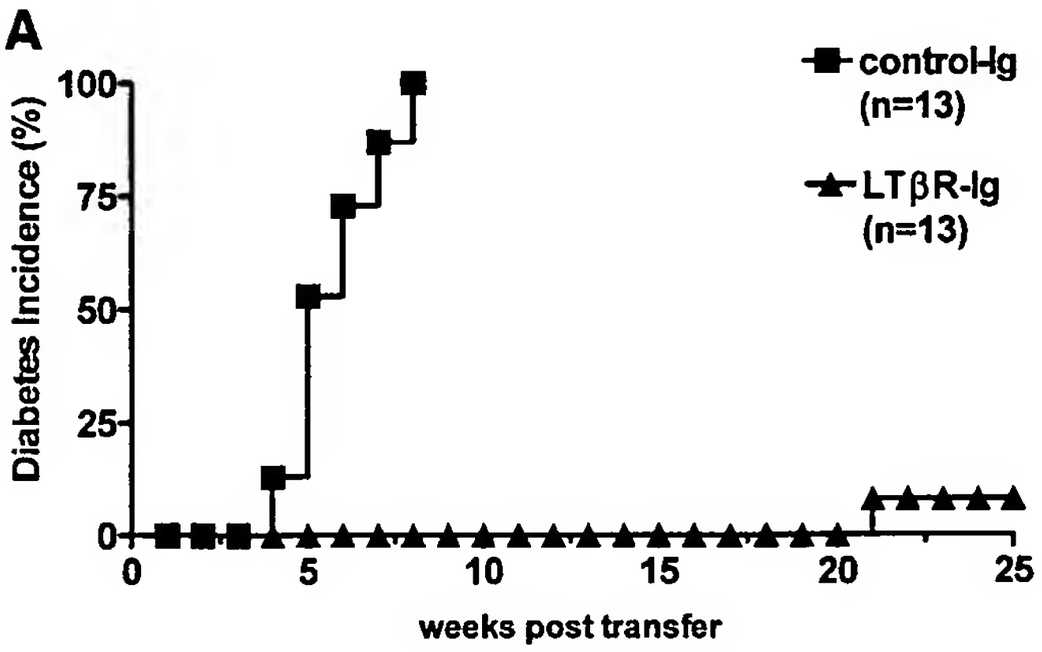

FIG. 4. The diabetogenic potential of splenocytes from nodeless mice was reduced. All NOD.scid recipients of splenocytes (2×10^7) from control immunoglobulin mice (13 of 13) developed diabetes by 8 weeks posttransfer. Only one (1 of 13) of the NOD.scid recipients of splenocytes (2×10^7) from nodeless NOD mice developed diabetes at 25 weeks posttransfer (*A*). Nodeless NOD mice were susceptible to the adoptive transfer of diabetes. The transfer of diabetic splenocytes (2×10^7) into irradiated control and nodeless NOD mice induced diabetes with similar kinetics and cumulative incidence (*B*).

ACKNOWLEDGMENTS

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Rheumatoid arthritis and its animal models: the role of TNF-α and the possible absence of specific immune reactions

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Rheumatoid arthritis is an organ-specific inflammatory disease of humans. Recent studies have focused on associations with non-MHC genes, new autoantigens and the role of innate immune responses. The success of anti-TNF-α in the majority (but, interestingly, not all) of patients has implications for disease mechanisms but the dangers of long-term therapy are becoming clearer. A number of new models of arthritis have been defined and emphasize the importance of the genetic make-up of the host. Attention has also focused on why the joint is a particularly vulnerable site for inflammatory responses.

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Abbreviations

IDDM	insulin-dependent diabetes mellitus
IFN-γ	interferon γ
RA	rheumatoid arthritis
SE	shared epitope
TNF-α	tumor necrosis factor α

Introduction

This review aims to discuss some recent progress at the interface of human and animal arthritis research, with a special focus on three questions where a comparative approach between human and experimental animal research appears particularly relevant. Firstly, what is the role of the innate immune system in arthritis and, more specifically, why is the joint and not other organs affected when the innate immune system is activated by TNF-α overexpression or by adjuvant challenge? Secondly, what is the role of specific, in particular autoimmune, reactions in arthritis and what evidence exists for the involvement of specific autoimmunity in human rheumatoid arthritis (RA) despite the relative failure to demonstrate relevant autoreactivities? Thirdly, what is the reason for the obvious therapeutic success resulting from blockade of one single cytokine — TNF-α — in many cases of RA and why, under the same conditions, do some 30% of RA patients respond very poorly to TNF-α blockade?

Human RA

Epidemiology and genetics

Epidemiological investigations of RA provide support for a considerable heterogeneity of the disease, for the contribution of specific immune reactions in at least a subpopulation of cases and for a probable involvement of some environmental triggering factors.

The main support for the involvement of specific MHC-class-II-dependent immune reactions in the pathogenesis is obviously provided from the association of RA with HLA-DR shared epitope (SE)-bearing alleles [1]. Despite the large literature on RA–MHC associations, major issues still remain incompletely resolved as shown by two recent reports. The first was a large population-based report on incident cases of RA and demonstrated that the contribution of HLA-DR alleles, including the SE to RA defined by the American Congress of Rheumatology (ACR) criteria, was rather limited (odds ratio 1.8 with the SE; odds ratio 3.5 with HLA-DR0404 homozygosity) [2•]. The second report, from Zanelli and co-workers [3], also questioned the primary role of the SE by demonstrating a more significant linkage of RA with HLA-DQ alleles in their population of recent-onset RA patients; however this view remains controversial [4]. Both sets of data add to the concept that RA defined by ACR criteria is a heterogeneous disease where MHC class II alleles constitute both susceptibility and severity factors.

Concerning the importance of non-MHC genes for susceptibility to and severity of RA, far less is yet known. There is, however, accumulating evidence that genetic heterogeneity in genes determining the function of cytokines such as TNF-α, IL-1 and IL-10 are of importance [4–7]. Of considerable interest — also for the discussion on genetic susceptibility to experimental inflammatory diseases — is the preliminary evidence for common genetic regions that influence the susceptibility to several different organ-specific inflammatory diseases such as RA, MS (multiple sclerosis) or IDDM (insulin-dependent diabetes mellitus) [8].

The possible involvement of environmental factors as triggers of the disease receives some circumstantial support from the fact that disease concordance in monozygotic twins is only around 15% (compared with 1% in the general population) [9]. Exposure to silica, to blood transfusions or to smoking are currently the best-documented examples of such environmental provocations [10] and a recent report provided additional support for an etiologic role of smoking in RA [11]. So far nothing is known of how these agents may exert their effects but the possibility that they may contribute to arthritis by means of nonspecific activation of inflammation and immunity will be discussed later in the context of the various animal models.

What evidence is there for a role of immunological specificity in RA?

Whereas the genetic evidence favors the existence of specific immunity contributing at least to some cases or some aspects of disease, the failure to reproducibly detect

autoimmune reactions to candidate autoantigens has remained an enigma in RA research. Three nonexclusive possibilities exist to explain the situation. Firstly, there may be no need for such specific immune reactions. This 'negative' possibility is obviously very difficult to prove in humans but will be discussed in depth in the section on animal models below. The second possibility is that relevant antigens remain to be investigated. Some support for this notion comes from recent studies where T cells from a subpopulation of RA patients responded more than controls to three different antigens: a newly described p205 antigen derived from synovial fluid [12•]; a cartilage-derived proteoglycan (aggrecan) [13•]; and a cartilage-derived protein termed gp39 [14]. Interestingly, the reactivity to the aggrecan was detected only when keratan sulphate side chains were removed from the native aggrecan [13•]. This latter observation suggests that modification of target antigens to produce cryptic determinants may be a possibility to consider in RA. As to gp39 and p205, it remains to be established to what extent these antigens are preferentially expressed in cartilage and thus to what extent autoimmunity to them should be considered organ-specific or systemic. The third possibility for the failure to detect responses to autoantigens is that reactivities to major cartilage-specific antigens such as collagen II may have remained undetected due to the pronounced downregulation of many T cell functions in RA patients (see below); preliminary support for this possibility exists in patients carrying the SE, at least for collagen type II [15].

The synovial inflammation

The synovial inflammation in RA is dominated by activated macrophages. Infiltrating T cells lack certain activation markers and in particular appear to produce low amounts of cytokines [16]. So far, little is known about which factors cause the extraordinary macrophage activation in joints. There is support for alterations in growth control, including p53-mediated events [17•] but the primary reason for this activation of the macrophage (a representative of the innate immune system) in the joint remains obscure. Concerning synovial T cells, their ability to respond to antigens is downregulated in several ways; they respond poorly both to TCR-dependent immune activation and to stimulation using alternative pathways [18,19]. It appears that overexpression of TNF-α may contribute at least to the downregulation of TCR-dependent T cell activation [20,21]. This would constitute a feedback situation where, for example, removal of TNF-α action might result in a *de novo* T cell activation — perhaps against different autoantigens.

Animal models of RA

The number of animal models of polyarthritis where clinical manifestations display similarities to those of human RA is steadily increasing. Interestingly, arthritis appears to be the result of a number of different challenges to the immune system — indicating that different forms of RA in humans may show features of different animal models. Challenges that can induce arthritis can broadly be divided into those that involve triggering of specific autoimmune reactions (mainly against cartilage autoantigens) and, more remarkably, challenges that are much more general but that, in several different cases, result in arthritis but not in other organ-specific inflammatory lesions.

Arthritis associated with autoimmune reactions to cartilage-derived molecules

The classical ways of triggering organ-specific inflammation in animal models, that is by immunization with organ-specific autoantigens emulsified in adjuvants, were for a long time mainly restricted to use of collagen type II. An increasing number of reports are, however, now demonstrating that several other cartilage-derived molecules are also arthritogenic provided that the immunized animals have an appropriate genetic constitution. Thus, arthritis has now been described after immunization with collagen II, IX [22] and XI, with cartilage oligomeric protein (COMP) [23•] or with the proteoglycan-associated molecules aggrecan [24•] and cartilage link protein [25•].

As to tolerance mechanisms and epitope recognition, it was demonstrated, firstly, that T cells reactive to the immunodominant epitope (amino acids 256–272) of collagen II in H-2q$^+$ mice were partly anergic, in the sense that they did not proliferate upon stimulation with collagen, but were nevertheless able to produce IFN-γ [26•]. Secondly, evidence was presented that the main arthritogenic T cell reactivity in collagen-induced arthritis (CIA) may indeed be against a glycosylated epitope whereas reactivities to other nonglycosylated epitopes do not contribute to disease [27•]. Taken together these two findings indicate, firstly, that T cell responsiveness to putative autoantigens in RA may not be possible to detect using proliferation assays and, secondly, that use of synthetic peptides may not be adequate to detect T cell reactivities to collagen II either in experimental arthritis or in human RA.

A molecule that may constitute an intermediate between a systemic and an organ-specific autoantigen is gp39, which not only is a potential autoantigen in RA but also has been shown to induce polyarthritis after being injected together with adjuvant into Balb/c mice. An interesting feature of the gp39-induced arthritis is that gp39 appears to be an almost ubiquitously expressed molecule which only under certain circumstances is overexpressed in the inflamed joint (see [14]).

As to genetics, the most interesting development has been the identification of several new non-MHC susceptibility regions in the collagen-induced arthritis model [28–31]. So far, the genetic regions are not defined in a way to permit the definition of specific susceptibility genes but two features of the findings deserve special notice: firstly, many of these genetic regions are the same as those defined in several other autoimmune conditions such as IDDM, EAE (experimental autoimmune encephalomyelitis), uveitis and others; secondly,

many of these regions are also identified in the genetic analysis of susceptibility to adjuvant arthritis. One possible explanation for these findings is that genes determining the response pattern to the adjuvant are of common importance for a number of different autoimmune diseases.

Arthritis induced by non-organ-specific stimulation

Arthritis induced by adjuvants

Classical adjuvant arthritis is one where a mixture of killed mycobacteria and mineral oil (Freund's complete adjuvant) induces arthritis in certain strains of rats after one single injection, usually given subcutaneously at the back of the tail. For a long time, the prevailing notion was that this arthritis is most probably caused by a crossreactivity between specific lymphocytes recognizing epitopes present both on the mycobacteria and on autoantigens in joints. This concept is, however, increasingly challenged by the findings that that adjuvant arthritis can be induced in certain strains of rats using a variety of simple adjuvants that do not contain any structures that are recognized by conventional T lymphocytes [32–34].

So far it has been possible to induce this adjuvant arthritis most easily in rats but much less easily in mice. However, it was recently demonstrated that mice also might develop adjuvant arthritis after neutralization of IL-4 with monoclonal antibodies *in vivo* [35] — indicating that the potential to develop adjuvant arthritis is indeed present in several species. Interestingly the susceptibility, severity and chronicity of the adjuvant arthritis in rats are regulated — partly independently of each other — by several non-MHC as well as certain MHC genes (for a review, see [36]). As indicated above, these genetic regions in many cases colocalize with regions that are also important for other inflammatory diseases such as experimental autoimmune encephalomyelitis, IDDM and others.

Because adjuvant arthritis is a T-cell-dependent disease, two main possibilities exist that may explain how an organ-specific inflammation in joints can result from provocations with simple adjuvants: firstly, T cells with some preference for binding to target molecules in joints are triggered by the nonspecific adjuvant stimulus but the specificities of these cells have so far remained undetected; secondly, no bias towards joint-recognizing T cells is present and instead joints are especially likely to be the targets for inflammation after certain types of immune activation that may encompass recognition of ubiquitous antigens. We cannot at present discriminate between these two possibilities although the latter possibility is accumulating support from the data now obtained in other models, as detailed below.

Increased TNF-α expression causes arthritis

A novel and unexpected finding made some years ago by Kollias and co-workers [37] was that transgenic mice overexpressing human TNF-α rapidly and spontaneously develop a severe ankylosing arthritis that is independent of the action of T or B cells. This observation immediately raised the question of why a potentially general overexpression of this cytokine caused an inflammation that was restricted to joints. However, further study of these TNF-α-transgenic mice, now on a DBA/1 background, demonstrated that the human TNF-α transgene was not expressed in resting macrophages or T and B cells of the spleen and lymph nodes but was preferentially expressed in cells of the synovial lining cell layer [38]. This, in turn, raises the question of why expression of this proinflammatory cytokine is preferentially induced in the joint — a question that is in some aspects very similar to the question of why the joints are targets in the general activation of macrophages, which is the primary event in adjuvant-induced arthritis.

Collagen II may cause arthritis without a contribution by specific immunity

Kollias and co-workers [39•] recently made an additional unexpected finding that may be of relevance for several models of arthritis, namely that injection of native collagen II (without any other additives such as adjuvants) into $Rag^{-/-}$ mice resulted in arthritis but not in inflammation elsewhere. This finding may indicate that native collagen II may by itself activate parts of the innate immune system without contribution from T or B cells and thus that collagen II naturally exposed to the synovial cells in the joints may exert such effects.

Systemic autoimmunity may trigger arthritis

A third, relatively recently described, arthritis model is the one where mice that were made transgenic for a TCR recognizing a bovine ribonuclease peptide presented by $I\text{-}A^k$, and alloreactive for $I\text{-}A^{g7}$-associated alloantigen, were crossed with the autoimmunity-prone nonobese diabetic to (NOD) mouse; to the surprise of the investigators, a polyarthritis developed in the absence of inflammation elsewhere [40]. The arthritis was proven to be destructive and chronic and showed a number of similarities both to the various induced models discussed above and to human RA. After first having shown that the arthritis was dependent on the activation of T cells against systemically available antigens, the group has continued its analysis of the model and has demonstrated that arthritis can be transferred by IgG from the TCR-transgenic mice [41••]. Recent evidence suggests that the target of the arthritogenic IgG reaction also appears to be a ubiquitous self-antigen. This model thus describes an additional situation where immunity — here B cell immunity in the effector stage — against ubiquitous antigens mediates development of arthritis, but not inflammation elsewhere, in genetically susceptible hosts.

The combined data from different animal models demonstrate that arthritis can develop secondarily to several different stimuli and with the use of several different effector pathways. If these experiences in animal models are also applicable to human RA, we might anticipate that several different types of infections as well as other environmental

exposures with adjuvant properties and with capacity to induce TNF-α production (for example the already identified risk factors for RA such as silica exposure and blood transfusion) may exert effects that in genetically susceptible individuals contribute to disease either together with some autoimmune reaction or by themselves.

Conclusions and therapeutic remarks

The use of TNF-α in RA

The growing suspicion that specific immunity is essential for disease development only in certain cases of arthritis has obvious therapeutic consequences and is in fact well in line with the rapidly accumulating empirical experience from therapeutic options that are currently tested clinically. Thus, the stunning success of TNF-α blockade in many cases of RA [42••,43••] is well in line with the evidence of an important role of TNF-α also in animal models. It is therefore of considerable interest to understand the mechanisms responsible both for the positive effects of TNF-α blockade in the majority of treated patients and the lack of effect in about one third of the patients. The difference in response suggests that these two subsets of RA patients may have different pathogenesis of their disease or at least activate different sets of cytokines leading to arthritis. A preliminary analysis of the cytokine pattern within synovial tissues of responders and nonresponders to TNF-α blockade has indeed indicated a relative lack of TNF-α in the synovial tissues of the nonresponders [44]. If confirmed in a larger study, this would provide an impetus to try blockade of other molecules such as IL-1 in those patients with low amounts of TNF-α in their joints.

The systemic effects of TNF-α blockade

Efficient blockade of TNF-α may obviously have large impacts on other immunological events than those contributing to RA. It is now known that members of the TNF family play a major role in the development and function of many parts of the immune system [45]. TNF-α-deficient mice fail to develop follicular dendritic cell networks, germinal centers and the normal maturation of the antibody response [46]. Studies utilizing TCR-transgenic mice have provided evidence that chronic exposure to TNF-α *in vivo* or *in vitro* decreases the T cell response to cognate peptide–MHC ligand whereas chronic exposure to anti-TNF-α either *in vivo* or *in vitro* results in an increased T cell response to cognate peptide–MHC stimulation [20]. These studies are paralleled by studies showing that TNF-α administration to adult (NZB x NZB)F1 mice and nonobese diabetic mice delays the onset and decreases the incidence of autoimmunity whereas administration of anti-TNF-α to adult animals of these strains increases autoimmunity [47,48].

Taken together, these findings suggest that chronic systemic TNF-α blockade may lead to heightened immune responses, as has already been observed in mice, with suggestive similar findings in patients treated with TNF-α blockade. This raises the possibility that long-term therapy of RA by means of TNF-α blockade might result in an increased incidence of other types of autoimmune diseases, depending on the complete HLA-DR and -DQ genotype of patients under treatment. Similarly, chronic TNF-α blockade might be expected to result in an increase in infections in which macrophages play a major role in systemic defense since blockade of TNF-α leaves the action of IL-10 unopposed and could lead to a relative deficiency of macrophages in immune defence [49,50]. For these reasons any plans to continue therapy with TNF-α blockade beyond relatively short-term and acute interventions should be carried out with great caution and careful monitoring, both for the development of autoimmune disease and for the development of those types of infections in which macrophages play a major role in defense.

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MINIREVIEW

COLLAGEN-INDUCED ARTHRITIS, AN ANIMAL MODEL OF AUTOIMMUNITY

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Summary

Collagen induced arthritis (CIA) is an autoimmune model that in many ways resembles rheumatoid arthritis (RA). Immunization of genetically susceptible strains of rodents and primates with type II collagen (CII) leads to the development of a severe polyarticular arthritis that is mediated by an autoimmune response. Like RA, synovitis and erosions of cartilage and bone are hallmarks of CIA, and susceptibility to both RA and CIA is linked to the expression of specific MHC class II molecules. Although not identical to RA, CIA clearly establishes the biological plausibility that an autoimmune reaction to a cartilage component can lead to a chronic, destructive, polyarthritis. Although it is induced in susceptible animals by immunization with heterologous CII, it is the autoreactive component of the immune response that leads to disease. A wealth of evidence indicates that synovitis is initiated by the production of pathogenic autoreactive antibodies capable of fixing and activating complement. The elucidation of the specific amino acid sequences of collagen that are recognized by the MHC molecules has enabled at least two approaches to specific immunotherapy to be considered. Firstly, small synthetic peptides representing dominant epitopes have been used as effectively as the original antigen as a tolerogen. The rather fastidious physicochemical properties of collagen that make it difficult for its routine use in therapy are thereby circumvented by the use of oligopeptides. Secondly, analysis of the specific amino acid side chains that are involved in MHC contact and TCR recognition enables analog peptides to be devised which can specifically

Key Words: collagen-induced arthritis, autoimmunity, T cell, cytokines, peptides

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and exquisitely inhibit the response to CII, preventing the onset of arthritis. Further investigations involving this model may contribute to the development of specific immunotherapies in the human disorder.

Introduction

Animal models of autoimmune diseases have been developed for a number of human disorders in the hope that the study of these models will lead to the development of effective and safe therapies. In most cases, these experimental diseases are induced by immunization with an antigen suspected of playing a role in the analogous human disease. In 1977 Trentham, *et al.*, reported that immunization of rats with type II collagen (CII) induced an autoimmune arthritis that in many ways resembled rheumatoid arthritis (RA) (1). Following immunization with CII, these rats developed an erosive, polyarticular arthritis mediated by an autoimmune response to the rat CII. Similarly, this same collagen-induced arthritis (CIA) model has been reproduced in the mouse (2) and in primates (3,4). The significance of this model is that CII is the major constituent protein of cartilage in diarthrodial joints, the predominant site of inflammation in RA. In addition, the pathogenesis of CIA is in many ways similar to that of RA. Histologically, both RA and CIA are characterized by an intense synovitis accompanied by erosions of cartilage and subchondral bone by a pannus-like tissue (5). Like RA, susceptibility to CIA in rodents is closely associated with the expression of specific class II molecules of the major histocompatibility complex (MHC) (6-8). Finally, CII has received considerable attention as a potential antigen in RA because immunity to CII can be detected in RA patients (9-14). In addition to serving as a valuable tool to study immunity to type II collagen, the CIA model has proven equally useful to investigate inflammatory joint injury. Unlike the models of adjuvant-and antigen-induced arthritis which are largely, if not entirely, mediated by T cells, CIA permits the study of synovitis induced by antibodies and propagated by T cells specific to a major glycoprotein found only in cartilage. Furthermore, the ease of inducing severe arthritis in highly susceptible strains of rats and mice add to the practicality of this model which has, and undoubtedly will continue to prove useful in dissecting the contributions that unique cell-types, cytokines and adhesion molecules play in the pathogenesis of the inflammatory arthritides that afflict man.

The autoimmune response to CII in the CIA animal models is complex, requiring specific major histocompatibility complex (MHC) molecules, CII-specific T cell and B cell immune responses and their associated cytokines, and several other cellular and biochemical functions (6,15-27). Unlike many animal models of autoimmunity, antibody plays a major role in the immunopathology of the autoimmune arthritis. The arthritis in the CIA model primarily affects the peripheral articular joints and is monophasic. Histologically, the inflammation initially develops as an acute synovitis, with erosions of bone and cartilage occurring shortly after. The arthritic joints heal slowly, often resulting in fibrosis and ankylosis of the involved joints (5). Many of these components of the CIA model have been studied in detail in the murine model, and in this review we will focus on those that define the autoimmune response and those that have been used as potential points of immunotherapy. In all, these studies have carefully defined the pathogenesis of CIA and have yielded significant clues as to potential mechanisms involved in the pathogenesis of RA.

Immune Response to CII in CIA

Major Histocompatibility Complex (MHC) Genes

Like many of the autoimmune diseases of man, including RA, susceptibility to CIA in the rodent models is linked to the expression of specific class II molecules of the major histocompatibility

complex (MHC). MHC class II molecules are highly polymorphic, integral membrane proteins, expressed as heterodimers. Their function is to serve as self recognition structures for the antigen specific T-cell receptor (TCR) (28,29) and as receptors for the binding and presentation of antigenic peptides to these T-cells (30,31). Both of these functions have a high degree of specificity that is controlled by the clusters of allelic polymorphisms located in the amino-terminal domains of both chains. In 1981 Wooley *et al.* demonstrated that susceptibility to CIA in the mouse is linked to expression of the class II isotype I-A, and specifically the I-A^q allele (6). Using a large panel of B10 congenic mouse strains they demonstrated that only H-2^q (I-A^q) mice were susceptible to CIA when immunized with chick CII, and this susceptibility was accompanied by strong T cell and B cell responses to the immunogen. However, the H-2^q strains of mice were not the only strains that developed a strong immune response to chick CII. Several other strains also produced high titers of CII-specific antibody, yet did not develop CIA. Thus, it has been hypothesized that the susceptibility to CIA in the mouse is dependent on the ability of the class II molecule to bind "arthritogenic peptide(s)" derived from CII and subsequently to stimulate CII-specific T cells capable of promoting an arthritogenic response. Equivalent observations have been made using inbred strains of rats which are more commonly susceptible to CIA than resistant (8).

Although initial studies utilizing chick CII as the immunogen identified only H-2^q mouse strains as CIA-susceptible, it was later determined that H-2^r mice were also susceptible to CIA when immunized with either porcine or bovine CII (7). The difference in this susceptibility is undoubtedly a result of the difference in the sequence of the CII molecules (bovine versus chick) and in the sequence (and thus function) of the I-A^r and I-A^q molecules. H-2^q mice (*e.g.* DBA/1 and B10.Q) develop a high incidence of arthritis following immunization with bovine, chick, and human CII, but not porcine CII (Table 1). On the other hand, H-2^r mice (*e.g.* B10.RIII) develop arthritis following immunization with bovine or porcine CII, but not with chick or human CII (32). These data imply that different T-cell antigenic determinants are involved in the induction of arthritis in these different strains of mice, and this is indeed the case.

Although immunity to CII has been detected in patients with RA, determining the relationship between immunity to CII and the role of the HLA class II alleles that confer susceptibility to RA has been difficult. The recent development of transgenic mice expressing HLA class II molecules has made it possible to address this problem experimentally. One such RA susceptibility allele, HLA-DQ8 (DQA1*0301 and DQB1*0302) was recently established as a transgene in mice causing a severe arthritis following immunization with bovine or chick CII (33). In a similar approach (DRB1*0101) was established as a transgene in B10.M mice, a strain genetically resistant to CIA (34). When these mice were immunized with human CII they developed CIA at ≥ 90% incidence, thus establishing that the DR1 molecule is capable of presenting antigenic peptides derived from

TABLE 1
Immunogenetics and Susceptibility to Murine CIA

Class II Haplotype	Strains[a]	Arthritogenic CII	CB Fragments that Induce Arthritis[b]
I-A^q	DBA/1, B10.Q, NFR/N	bovine, chick, human	CB11
I-A^r	B10.RIII	bovine, porcine	CB8

[a]Data are compilation from Wooley *et al.*, (6,7), and Holmdahl *et al.* (75).
[b]Arthritogenicity of CB11 fragment has been reported for chick and bovine CII (40,41); CB8 arthritogenicity is based on bovine CII, as described by Myers *et al.* (48).

human CII (35). Like CIA in non-transgenic mice, the arthritis is associated with development of a T cell response and autoantibodies to CII. Although these data by no means indicate that immunity to CII initiates the pathology of RA, they support the hypothesis that the part of the pathology of RA includes an immune response to autologous CII. These transgenic approaches to understanding the function of the class II susceptibility genes in RA hold great promise for advancing both our understanding of the immunopathological mechanism of RA as well as bringing us a step closer to the development of modern immunotherapies.

In contrast to class II genes that confer susceptibility to CIA, several class II molecules have been described that confer protection from developing CIA. The presence of $E\beta^d$, $E\beta^s$, and $I\text{-}A^b$ murine class II molecules, as well as the addition of an HLA-DR2 transgene to CIA susceptible mice, have all been shown to protect genetically susceptible mice against the development of CIA (36-39). These class II molecules appear to confer protection by serving as a source of peptide that binds to the susceptible class II molecule, thereby altering its function in terms of availability to present other peptides or in the alteration of the T cell repertoire via thymic selection.

T Cell Determinants

Attempts to identify antigenic peptides presented by $I\text{-}A^q$ and $I\text{-}A^r$ were initially hampered by the large size of the CII molecule. Structurally CII is a homotrimer of $\alpha 1$(II) chains, composed of 1018 amino acids. To simplify its analysis, initial studies focused on the use of cyanogen bromide (CB) digests of CII to identify fragments capable of stimulating T cells and inducing CIA. In $H\text{-}2^q$ mice, only one large fragment, cyanogen bromide peptide 11 (CB11, residues 124-402) was found to induce a T cell proliferative response and elicit CIA (40,41). Although the incidence of arthritis induced by CB11 immunization was decreased in comparison to immunization with native CII, histologically it is very similar to the autoimmune arthritis induced with native CII. Detailed analysis of the CB11 antigenic determinants revealed an immunodominant T-cell determinant, CII(260-267), that stimulated both T cell proliferation and cytokine production (Figure 1) (41-44). Following immunization with CII, H-2 congenic strains of mice that are not susceptible to CIA do not mount a T cell response to this determinant, whereas cells from disease-susceptible B10.Q and DBA/1(both $H\text{-}2^q$) mice respond strongly (45), establishing the importance of this determinant. Interestingly, four additional subdominant $I\text{-}A^q$ restricted T cell determinants have been identified within CB11 (Table 2), CII (190-200), identified in chick CII by lymphokine production assays (45,46); CII (224-243) identified in human CII by T cell proliferation assays (44); CII (295-303) identified in chick CII by both T cell proliferation and lymphokine production assays (44,45); and CII (384-402) identified in chick CII by lymphokine production assays (45). The BB rat similarly responds to peptide CII (290-200) with a strong T cell proliferation (47). Although proximate the epitope recognized by the BB rat is unique from the epitope recognized by DBA/1 mice. These T cell determinants may play additional roles in inducing arthritis, modulating disease, or maintaining chronicity.

In contrast to $H\text{-}2^q$ mice, $H\text{-}2^r$ mice develop T cell immune responses to two CB fragments of bovine CII, CB8 (403-551) and CB10 (607-621) (48-50). Despite the fact that the CB10 determinant is clearly the more potent in terms of T cell proliferation, CIA can only be induced in $H\text{-}2^r$ mice by immunization with the CB8 portion of bovine CII (48). Analysis of the T cell determinants within both of these CB polypeptides again identified only a single antigenic determinant within each capable of stimulating T cell proliferation. A comparison of these T cell determinants revealed that they are remarkably similar, differing by only 2 amino acid residues throughout their determinant cores (Table 3). Since both determinants stimulate T cells, yet only one promotes the development of CIA, these data imply that only certain T cell responses can promote the development of the autoimmune arthritis. Finally, it is interesting to note that chick CII, which is non-

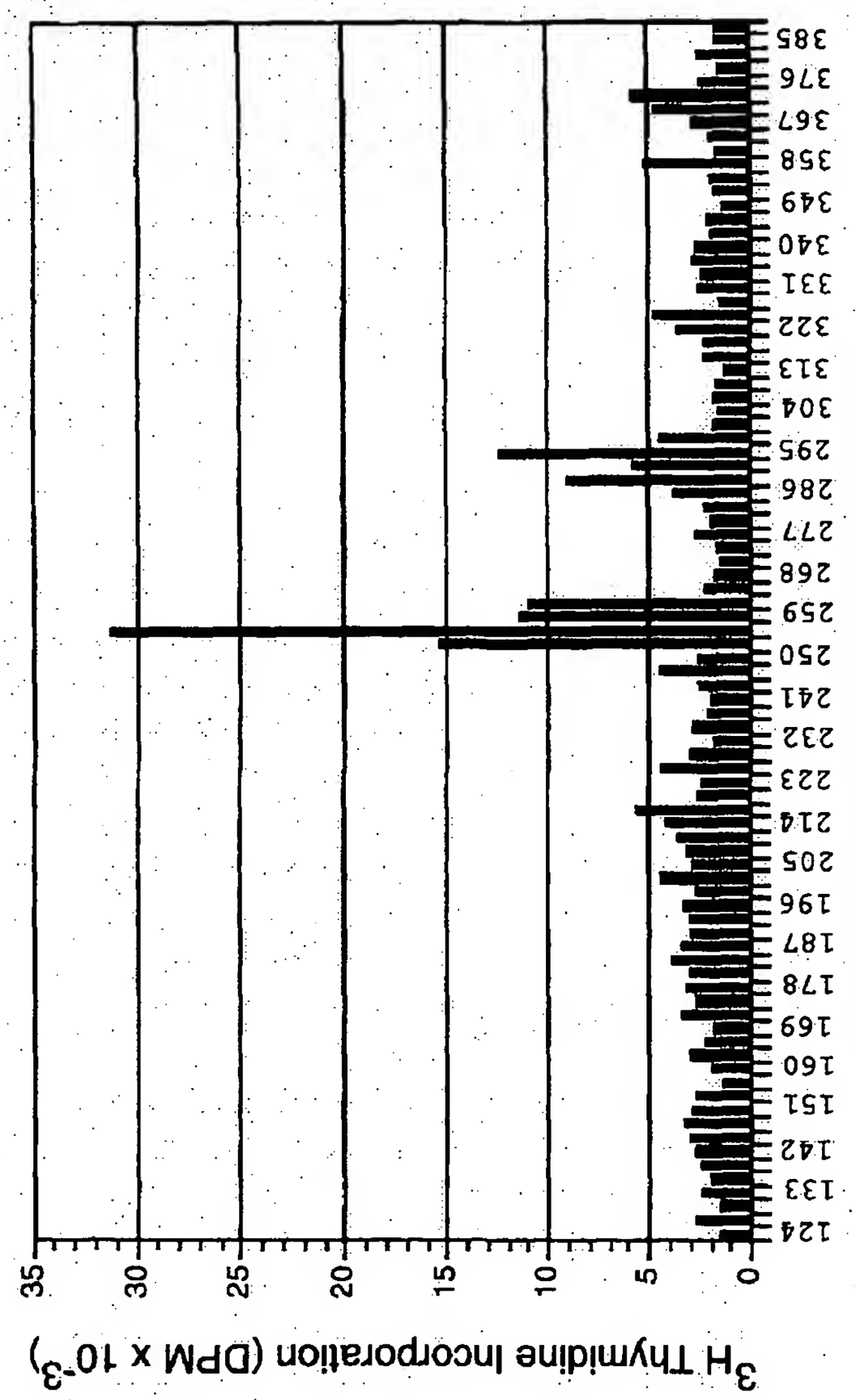

FIG. 1

Identification of I-A^q-restricted T-cell determinants within the CB11 portion of chick CII. DBA/1 mice were immunized with chick CB11 and draining lymph node cells were tested for their ability to be stimulated by a series of Mimotope peptides. Mimotope peptides were synthesized as 15-mers, overlapping by 12 amino acids, and spanning the entire length of chick CB11. Numbers on the abscissa identify the amino terminus of the chick CII peptide, and data are expressed as decays per minute (DPM).

TABLE 2
I-A^q Restricted T Cell Determinants Identified within CB11

I-A^q-Restricted Peptide[a]	Amino Acid Sequence
Chick CII(190-200)	G P R G E S G T B G S
Human CII (224-243)	A P G I A G A P G F P G P R G P P G P Q
Chick CII(260-270)	I A G F K G E Q G P K
Murine CII (260-270)	I A G F K G D Q G P K
Chick CII(295-303)	G E B G A A G P V
Chick CII(384-401)	D G R B G P B G P Q G A R G Q B G V

[a]Sequences for the five unique I-A^q T cell determinants that have been identified by various investigators are shown here. Previous studies utilizing overlapping synthetic peptides have identified one unique determinant generating both the greatest proliferation and the greatest T cell lymphokine responses, CII (260-267) (41-44) as well as four additional T cell determinants, CII (190-200) (45,144), CBII (224-243) (44), CII (295-303) (44,45), and CII (384-402) (45).

arthritogenic in H-2^r mice but can induce tolerance, contains a Val residue at position 450 in comparison to the Ala in bovine CII (Table 3). This difference apparently is critical in determining the arthritogenicity of these two collagens in H-2^r mice.

Since CIA is a model of autoimmunity, do T cells specific for murine CII play a significant role in the development of CIA? This question has been difficult to answer. While immunization with heterologous CII may break thymic tolerance to murine CII and initiate an autoimmune T cell response, it is equally possible that peptides derived from a highly tissue specific protein such as CII may never reach the thymus, allowing for the maturation of CII autoreactive T cells. When immunized with heterologous CII, both H-2^q and H-2^r CII-specific T cells cross react to a limited

TABLE 3
I-A^r restricted T Cell Determinants Identified Within Bovine CB8 and CB10

I-A^r-Restricted Peptide[a]	Amino Acid Sequence	Stimulatory Properties
Bovine CII(445-453)	G P A G P A G E R	Proliferation
Chick CII (445-453)	G P A G P V G E R	
Bovine CII(610-618)	G P A G T A G A R	Proliferation

[a]Amino acid sequence of the arthritogenic T cell epitope in bovine CB8, CII(442-456) (48), the nonarthritogen chick CB8 determinant (CII 442-456) (48). and the tolerogenic epitope in CB10, CII(607-621). Despite its ability to induce proliferation, CB10 does not elicit CIA in B10.RIII mice, although it is an effective tolerogen in these mice (49,50). Underlined residues indicate the amino acid sequence differences in these determinants.

degree with murine CII homologs. Immunization of H-2^q mice with murine CII does elicit CIA, however the incidence is substantially lower and the magnitude of the autoimmune response is diminished in comparison to immunization with heterologous CII (51). The murine homologue of the CII(260-267) immunodominant determinant clearly binds to the I-A^q molecule and is capable of stimulating T cells primed with bovine CII (52). The only difference in the sequences of the heterologous CII(260-267) (bovine or chick) and the murine CII(260-267) is a conservative substitution of an Asp (mouse) residue for a Glu (bovine and chick) residue at position 266 (Table 2), identified as a T cell interaction site in the antigenic peptide (52). Despite this conservative difference (change only in length of side chain), approximately half of I-A^q-restricted T-cell hybridomas specific for bovine or chick CII(260-267; Glu at 266) do not recognize the murine CII(260-267; Asp at 266) peptide (E. Rosloniec, unpublished observations).

Insights into the structural characteristics of the I-A^q molecule that confer susceptibility to CIA have been gained by comparison of the function and sequence of the I-A^q class II molecule with that of the very similar I-A^p class II molecule. The I-A^p molecule does not confer susceptibility to CIA, yet differs from I-A^q by only four amino acids (53). That these four amino acids alone control the difference in susceptibility between H-2^q and H-2^p mice was demonstrated by the production of a transgenic H-2^p mouse expressing a mutated genomic clone of I-A^p in which the four residues were converted to the I-A^q sequence (54). These four amino acids, residues 85, 86, 88, and 89, are all located within the fourth polymorphic region of the Aβ chain (53), and make a contribution to the formation of the peptide binding pocket located within this region of the class II molecule. This is especially true of the contribution of the amino acid at position 86 of the Aβ chain. The binding of the immunodominant CII(260-267) determinant to I-A^q is dependent largely on two amino acid residues, Ile at 260 and Phe at 263, that serve equally as primary anchor sites (52). Although I-A^p is also capable of binding and presenting the CII(260-270) determinant, the affinity of this peptide for I-A^p is much lower than the affinity for I-A^q (E. Rosloniec, unpublished observation). Presumably this is a result of the inability of the Ile at position 260 in CII to fit within the binding pocket defined by the Aβ^p residues 85 through 89. Therefore, it appears that differences in CII peptide binding affinity for I-A molecules also plays a major role in conferring susceptibility to CIA.

The fact that susceptibility to CIA is linked to expression of class II molecules of the MHC implies an important role for T cells in mediating the autoimmune response, and several studies support this viewpoint. Both treatment of rats with antiserum to thymocytes and mice with anti-CD4 has been shown to prevent the development of CIA (55,56). More recently, Yoshino and coworkers have demonstrated that severe depletion of α/β T cells by monoclonal antibody treatment reduces the incidence and severity of CIA in rats (57), and that greatly reducing the TCR repertoire genetically also reduces the severity of CIA in H-2^q susceptible mice (58,59). These studies confirm that CD4$^+$ cells are important in the induction phase of disease, and likely secrete inflammatory cytokines as well as promote the production of arthritogenic autoantibodies which together activate synovial macrophages and chondrocytes leading to the local release of pro-inflammatory cytokines and pannus formation. Although attempts to induce CIA by T cell transfer in mice have been largely unsuccessful (18), intrasynovial injection of cloned T cell lines induced microscopic evidence of arthritis (60).

It is curious to note that H-2^q mice in which CD4 has been genetically deleted show no decrease in susceptibility to CIA (61). Both incidence and severity of arthritis are unaffected. In comparison, genetic deletion of CD8 expression significantly reduced the incidence of arthritis. Although seemingly conflicting with the anti-CD4 data discussed above, the CD4$^-$ T cells in these "knockout" mice function as class II restricted T cells, and appear to fill the role of the absent CD4$^+$ T cells. This is apparently not an option for the immune system in mice that normally ex-

press CD4. Regardless, it appears that $CD4^+$, $CD8^-$ T cells from CD4 deficient mice can still produce the appropriate cytokines and provide the necessary help for promoting the production of arthritogenic antibodies. Fewer studies have addressed the role of γ/δ T cells in CIA. These cells have been found in synovial tissue and have been implicated to play a regulatory role in modulating the severity of CIA (62,63). An intraperitoneal injection of anti-TCR γ/δ mAb initiated one day before the injection of type II collagen significantly delayed both the onset and the severity of CIA, while its injection 40 days after the collagen injection resulted in a rapid onset of severe arthritis (62). Another important role of T cells in CIA is their production of inflammatory cytokines as a result of CII immunization. TH1 T cells in particular, promote the release of pro-inflammatory cytokines and/or the production of arthritogenic antibodies (64,65). Therefore (and not surprisingly), the susceptibility to CIA appears to be tightly regulated by T cell cytokines, confirming that T cells are important in CIA.

B Cell Response

The destructive autoimmune arthritis characteristic of CIA cannot be induced by immunization with short synthetic peptides containing only T cell determinants (L.K. Myers, unpublished observation). High levels of circulating autoantibody to murine CII invariably accompany the development of CIA and appear to be required for the development of disease. Several studies have demonstrated that autoantibodies to CII initiate joint inflammation by binding to articular cartilage and activating hemolytic complement (66). This deposition of IgG antibodies and the complement component C3 on the cartilage surface immediately precedes the development of overt arthritis (66). In addition, CIA can be passively transferred to naive mice of both susceptible and non-susceptible strains by CII-specific polyclonal antibodies purified from the sera of arthritic DBA/1 mice (67-70) and by a cocktail of monoclonal antibodies specific for CII (71). Passive transfer of CII-specific antibody induces a severe arthritis that develops within days of the transfer, although the arthritis is transient and fails to achieve chronicity. The fact that denatured CII and its fragments induce less severe disease than native CII may be due to a less efficient induction of an antibody titer which can cross-react with native murine CII. The importance of complement in the initiation of synovitis was described early in the history of the CIA model by Morgan, *et al.* (72), who demonstrated that CIA could be abrogated in actively immunized rats by administering cobra venom factor shortly before the predicted time of arthritis onset. Subsequent studies by Watson, *et al.* (69,73), corroborated the importance of complement by showing that CIA-susceptible $H\text{-}2^q$ mice, congenitally deficient in the C5 complement component, were resistant to both CII-induced and passively transferred arthritis. Thus these studies have established CII-specific antibody and complement fixation as the major immune mechanism of pathology in the CIA model (74).

Although it is clear that the production of antibody to CII is a major factor in determining susceptibility to CIA, it has also been established that some CIA-resistant strains of mice also produce appreciable quantities of CII-specific antibody (6,75). These data indicate that not all CII antibodies are capable of inducing arthritis, suggesting that there is a qualitative difference in the antibody response of susceptible strains. For example, congenic strains of mice, such as B10 ($I\text{-}A^b$) and B10.D2 ($I\text{-}A^d$), produce appreciable quantities of anti-CII antibodies following immunization with CII, which recognize predominantly the CB11 (CII 124-401) region of the CII molecule. These antibodies bind to the cartilage (76) but the mice do not develop arthritis (6,7,19). Possible differences in these antibody responses include the topographical distribution of binding sites recognized by the autoantibodies, the isotype/subclass composition, and their avidity of binding. In recent studies by Brand *et al.*, the determinant specificity and isotype of these autoantibodies were analyzed (77). Affinity-purified antibodies specific for murine CII were prepared from both CIA susceptible and nonsusceptible B10 congenic strains of mice immunized with CB11 (arthritogenic in $H\text{-}2^q$ mice), and their determinant specificity was compared (Figure 2). Although overall re-

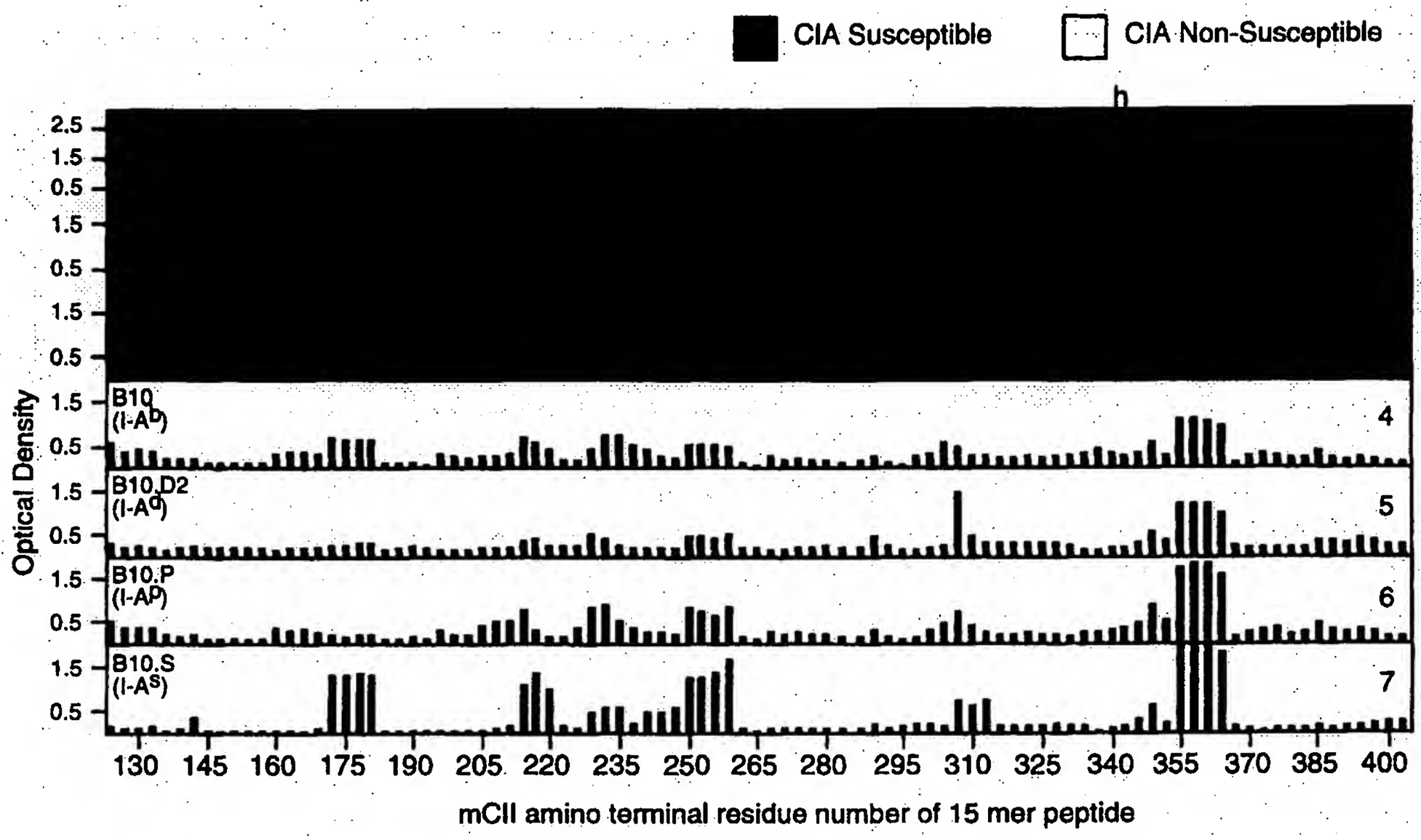

FIG. 2

Comparisons of autoantibody epitopes recognized by sera from CB11 immunized mice. Murine CII-specific antibodies were affinity purified from sera obtained from CB11-immunized mice and tested for their ability bind to a panel of Mimotope peptides covalently linked to pins. The Mimotope peptides were 15-mers, overlapping by 12 residues, and spanned the entire length of CB11, CII(124-402). Epitopes were identified using a modified indirect ELISA with an HRP-labeled anti-IgG Ab as a secondary reagent. Uppercase letters correspond to epitopes found in immune serum from more than one B10-congenic strain. Lowercase letters correspond to epitopes found in DBA/1 immune serum. Numbers on the abscissa identify the amino terminus of the murine CII peptide. Reproduced with permission from the Journal of Immunology (77). Copyright 1996. The American Association of Immunnologists.

markably similar, a unique subpopulation of autoantibodies that bound to a specific determinant was identified only in CIA susceptible B10.Q, B10.QbBR and DBA/1 mice, and the complement fixing sub class IgG2 was present in these populations of antibodies (77). These data suggest that the topographical distribution of antigenic determinants recognized by the autoantibodies may be important in initiating CIA.

As would be expected, activation of the complement cascade by CII antibodies initiates a synovial infiltration by inflammatory cells. The earliest cell type identified in abundance in the synovium is the neutrophil which is gradually replaced by infiltrating monocytes and lymphocytes (5). Undoubtedly the presence of the neutrophils in early lesions is a result of the strong chemotactic property of the complement product C5a. The first direct study to establish a role for neutrophils in CIA was performed by Schrier *et al.* (78) who observed that paw swelling in rats with established CIA was reduced by 51% after treatment with rabbit anti-rat neutrophil antibodies. This work has been recently confirmed by others (79). In addition, neutrophil deficient C57Bl/beige mice, are highly resistant to passively induced CIA (69). Neutrophil infiltration and function has become the focus of several studies focused on the design of novel therapies, including the suppression of CIA with monoclonal antibodies to the adhesion molecule ICAM-1 (CD54) (80), and the role of granulocyte-macrophage colony stimulating factor in CIA (81). Studies implicating other inflammatory cells include the attenuation of CIA with either antibodies to hyaluronate binding CD44 receptors (82,83) or (LAF)-1 (81,84-86). Finally, the potential role of the mast cell remains a topic of considerable interest in CIA but has only been casually explored. Studies implicating IgE anti-CII antibodies in CIA (87) plus the abundance of enzymes and proinflammatory mediators found in these unique cells make them an appealing subject for further investigation.

Cytokines in the Pathogenesis of Collagen-induced Arthritis

TNF-α

TNF-α not only accelerates arthritis in passively-immunized rats when administered intra-articularly, but does the same when given systemically to actively-immunized animals (64,88,89). Serious interest in cytokines as targets for biological interventions have been spurred by observations that intra-articularly injected cytokines produce acute synovitis (64,90), that TNF-α and its receptor are found at the cartilage-pannus junction (91), and that arthritis appeared spontaneously in transgenic mice expressing TNF-α (92). As anticipated, antibodies to TNF-α (88,93,94) or soluble TNF receptors (sTNFR) (93) have therapeutic effects when given to mice either shortly before arthritis onset or after it is established. A report by Wooley *et al.* (95) shows that CIA can be inhibited with a soluble recombinant TNFR Fc fusion protein in which the TNF receptor was fused to the Fc portion of an antibody and, unlike the previous studies using anti-TNF-α antibodies or sTNFR, antibody levels to CII were reduced in both preventative and therapeutic protocols.

IL-1

Considerable interest has been directed at exploring the role of IL-1 in CIA. This interest stems from the observations that IL-1 induces synovitis after intra-articular injection (96) and that anti-IL-1 antibodies prevent the spontaneous arthritis seen in TNF-α transgenic rats. These data suggest that TNF-α works via IL-1 release (97). The systemic administration of IL-1β, like TNF-α, increases the incidence and severity of CIA in DBA/1 ($H\text{-}2^q$) mice, and accelerates the time of arthritis onset (98,99). The timing of IL-1 administration appears to be of importance inasmuch as greater effects were noted when IL-1β was given shortly before the onset of disease versus when given within a week after immunization (98,99). Similar observations have been made in inbred WF rats where IL-1 given (100) on days -1 to +3 relative to immunization decreased paw swel-

ling, whereas IL-1 administration on days +6 to +10 worsened swelling, while IL-1 on days 13 to 17 had no effect (100). Caccese *et al.* (101) have demonstrated that CIA can be elicited in 85 to 90% of DBA/1 mice within 48 hr of a single intraperitoneal injection of LPS without using exogenously prepared IL-1. This same technique has proven effective in markedly reducing the amount and number of monoclonal antibodies required to passively transfer arthritis to naive mice (102).

The practicality of suppressing IL-1 as a therapeutic approach in treating arthritis is supported by the observation that CIA can be attenuated in mice by using a variety of biologic agents. Wooley *et al.* (103) found that daily injections of recombinant interleukin-1 receptor antagonist protein (rIRAP) beginning two weeks after immunization significantly reduced the incidence of CIA in mice in a dose-related manner. Antibodies to IL-1 have a similar effect on CIA in mice. Using a murine monoclonal antibody to IL-1β, Geiger *et al.* (104) were able to totally prevent CIA in DBA/1 mice when the blocking antibody was administered from day +3 after immunization with CII to day +60. In comparison, administering the antibody at a later time (day +21 to +60) was also effective but only reduced the incidence of arthritis. Van den Berg *et al.* (105) made similar observations and also found that the combination of polyclonal antibodies to IL-1α and β given before the onset of arthritis prevented disease and when given later reduced the severity of established arthritis. Likewise, antibodies to IL-1β administered alone provided significant benefit in contrast to antibodies to IL-1α which were minimally effective.

Interferon-γ

Interferon-γ, a TH1-type cytokine, has been proposed to play a major role in the induction of inflammation. Immunolocalization studies have provided evidence that this and other pro-inflammatory cytokines localize around inflammatory infiltrates and appear to function in the pathogenesis of certain autoimmune diseases (106), including CIA (107). Interferon gamma (IFN-γ,) for example, clearly exacerbates CIA when administered early in the course of the disease. An early study disclosed that moderate doses (2×10^4 U), given thrice weekly for four weeks following CII immunization, hastened the onset of arthritis in mice and increased arthritis incidence without affecting antibody levels (65). Boissier and co-workers (108) treated mice either with IFN-γ or a mAb to IFN-γ . They showed that 8×10^4 U of IFN-γ given thrice weekly starting at immunization worsened disease and early treatment with anti-IFN-γ mAb suppressed arthritis. Comparable results have been obtained by others using anti-IFN-γ during early disease (109). The effects on established arthritis are less clear, with reports of the onset of arthritis delayed and severity and antibody levels reduced when IFN-γ was given later (day 20 to 55) (108). Giles and co-workers (110) demonstrated that the proinflammatory action of IFN-γ is not absolutely necessary for the development of CIA in that H-2^q mice with a disruption of the IFN-γ receptor gene developed arthritis comparable to immunized DBA/1 controls .

The effects on CIA of other members of the interferon family have not been well studied. Perhaps the most intriguing report regarding the IFN family is by Yoshino (111) who found that 25×10^3 U of IFN-α/β given orally for five days before immunization reduced the severity of CIA in rats and suppressed DTH and T cell proliferative responses to CII. Unfortunately, the treatment was not effective on established disease. Recently another cytokine, IL-12, has been reported to enhance the severe joint disease of CIA, causing CII-specific T cells to produce greater amounts of IFN-γ which upregulates the synthesis of complement fixing IgG2 antibodies (112,113).

TH2 Cytokines: IL-4 and IL-10

T_H2 cytokines, such as IL-4 and IL-10 downregulate T_H1 functions in mice (114,115). An *in vivo* role of T_H2 cytokines in CIA is suggested by Mauri *et al.* who studied lymph node cytokine pro-

duction when CIA was in remission (116). They found that IL-10 was easily detected late in disease along with low, but persistent, levels of IL-4; at the same time IFN-γ production had declined. Mice given IL-4 parenterally developed CIA at a lower incidence than controls (87). Similarly, mice given daily intraperitoneal injections of murine recombinant IL-10 also develop significantly milder CIA at a lower incidence than controls (117). Bessis and coworkers demonstrated a decrease both in incidence and severity of CIA in groups of mice treated with either IL-13 or IL-4 gene-transfected CHO cells (118). It thus appears that T_H2-type cytokines can attenuate the inflammation of CIA while T_H1-type cytokines support inflammation (119,120).

TGF-β

The role which TGFβ plays in arthritis may at first appear paradoxical. Although capable of inhibiting B and T cell proliferation, TGFβ produces synovitis when injected into the joints of normal rats (90) and accelerates the onset of CIA when injected in to the joints of rats actively or passively immunized with CII (64). These actions have understandably been attributed to TGFβ's potent chemoattractant property for neutrophils and its ability to stimulate synovial fibroblasts vigorously. On the contrary, TGFβ suppresses CIA in mice when injected systemically before the onset of arthritis, especially when treatment is begun shortly after immunization with CII (88,89). By following these protocols, TGFβ reduces both joint injury, as judged by histology, and antibodies to CII; established arthritis also responds to late TFGβ therapy but less avidly. As perhaps anticipated, antibodies to TGFβ accelerate the onset of CIA (88).

Experimental Therapies in CIA

Induction of Tolerance

It is of great interest for development of potential therapies that arthritis can be prevented in mice when CII is given as a tolerogen prior to immunization. Like most antigens, tolerance to CII can be induced in mice via a variety of routes of administration including intravenous (42,68,121,122), oral (123,124), nasal (125,126), and intraperitoneal (45). CII is an excellent tolerogen whether given as soluble CII (121), or as CII-coupled spleen cells (127), CNBr peptides (128), or synthetic peptides (45,129). Mice tolerized by any of these approaches have decreased T cell responses, significantly lower antibody titers to CII, and significantly decreased incidence of CIA (42,68,122,130). Delayed intravenous injection of collagen can suppress arthritis up to 3 weeks after initial immunization with CFA + CII (122). Investigations of the mechanism of tolerance in CIA have focused on both the identification of structural determinants responsible for the suppression of arthritis and delineation of ways in which the T cell response to collagen has been altered. As might be expected, the I-A^q-restricted immunodominant determinant of CII is an efficient tolerogen in H-2^q mice (42), as is the dominant determinant in H-2^r mice (49,50). The induction of oral tolerance to autoantigens has become an area of intense investigation with the last few years. It is an attractive therapeutic approach because of its antigen specific effect as well as the ease of administration. Clinical trials using chick CII as an oral tolerogen for RA patients have been reported with some evidence of success (131,132). Oral tolerance has been shown to be quite effective in preventing autoimmune disease in several animal model studies (133), including CIA (123). Mechanistically, the induction of tolerance to CII appears to change the response of CII-specific T cells by alter their cytokine production. When spleen cells obtained from mice tolerized with CII were stimulated by CII *in vitro*, the levels of IL-4 and IL-10 were significantly increased (126), as well as reduction in the levels of the complement-fixing IgG2 antibodies specific for CII in comparison to controls (125,126). Thus, it appears likely that tolerance to CII induces T cells to secrete TH2-type cytokine profiles which modulate inflammation and markedly decrease incidence and severity of autoimmune arthritis. CIA is an excellent model to test and develop mecha-

nisms of tolerance which may be applicable to human disease, provided the human autoantigens can be identified in the future.

Altered Peptides

Another recently described approach to antigen specific immunotherapy of autoimmune diseases is by means of the altered peptide ligand (APL) (134,135). APL are analog peptides of T cell antigenic determinants in which residues important for antigen presentation have been altered, and the analog peptide now functions as either an antagonist or partial agonist of the TCR. APL have been shown to effectively inhibit both MHC class I (136) and class II (134,137) mediated responses, as well as the autoimmune response of CIA (138). A synthetic peptide analog of the I-A^q-restricted CII(260-270) determinant containing three amino acid substitutions (residues 260, 261, and 263), has been found to quite effective in blocking the T cell response to CII, and when co-administered with CII, preventing the onset of arthritis (138). More importantly, this analog peptide is capable of down regulating the immune response to CII and the incidence of arthritis when given as much as 3 weeks after immunization with CII (L.K. Myers, unpublished observation).

Although the exact mechanism whereby this analog peptide affects arthritis has not been completely elucidated, our hypothesis is that this inhibition is mediated through a T cell receptor (TcR)-based phenomenon. Because of the amino acid substitutions, the analog may have altered the affinity of its binding to the major histocompatibility complex (MHC) class II molecule or the TcR recognition site of T cells which ordinarily react with the immunodominant CII 260-270 bound to I-A^q. The resulting interaction with the TcR delivers only a partial signal, failing to induce effective signal transduction. The fine specificity of major histocompatibility complex (MHC)-peptide recognition by the TcR may have been altered so that differential signaling results in two potential outcomes. In one scenario, by means of an alteration in signaling through the TcR, the analog may induce T cells to produce an altered profile of lymphokines, the majority of which are suppressive-type lymphokines capable of down-regulating the production of inflammatory lymphokines and autoantibodies which induce and exacerbate autoimmune arthritis. Alternatively, the analog may function as a TcR antagonist, severely inhibiting T cells which ordinarily react with CII 260-270, causing them to become completely anergic and refractory to subsequent stimulation. The use of APL in the treatment of autoimmune disease is receiving considerable attention as the evidence of their efficacy continues to grow in *in vitro* studies as well as in animal models. However, once again, the development of such therapeutics for human diseases relies upon significant knowledge of the autoantigen.

Targeting T Cells

Like most antigen specific T cell responses, there is a restrictive repertoire of T cells that respond to the antigenic determinants in CII (139). The TCR is a heterodimer in which each chain is composed of a constant region (Cα and Cβ) and a variable region (Vα and Vβ). Analysis of the Vα and Vβ regions utilized by the I-A^q-restricted TCR specific for CII identified both conserved usage of Vα (Vα8, Vα11, and Vα22) and Vβ (Vβ8, Vβ1, and Vβ6) chains (26). Considering the powerful diversification mechanisms available for the TCR α and β chain genes, it is apparent from theses data that the CII antigen selects a restricted repertoire for its recognition. This limited repertoire has become the focus of several studies attempting to develop specific immunotherapies in the CIA model. One approach has been the use of monoclonal antibodies specific for the Vβ chains of these TCR. Injection of monoclonal antibodies specific for Vβ6 and Vβ8.2 prior to CII immunization has been used successfully in preventing CIA development in the DBA/1 (H-2^q) mouse (59,139-141). Although the antibody treatment is effective, it's protection is not long lasting. The antibody eventually degrades and the T cells re-emerge. An alternate approach to T cell specific therapy has been the use of TCR-based vaccinations. TCR vaccinations have been used

successfully in the CIA model (142) as well as other models of autoimmunity (143) to specifically alter the T cell response. In the CIA model, a recombinant expressed Vα11.1-Jα17 protein was used to vaccinate DBA/1 mice prior to immunization with CII. This TCR chain was derived from a T cell specific for the CII(260-270) immunodominant determinant of CII. The vaccination significantly reduced the incidence of CIA, and induced both a T cell and B cell response to the TCR chain. Although both of these approaches have potential in the treatment of autoimmune diseases, they both require significant knowledge of the initiating antigen, an unknown in most human autoimmune diseases. The fact that more than one TCR can drive CIA suggests that use of more than one TCR peptide may be potentially more efficient.

In conclusion, CIA clearly establishes that an autoimmune reaction to a cartilage component can lead to a chronic, destructive, polyarthritis. Besides serving as a valuable tool to study immunity to type II collagen, the CIA model has proven equally useful to investigate inflammatory joint injury. As newer technologies are developed to analyze the immune system, this model will undoubtedly continue to prove useful in dissecting the contributions that specific molecules and immune components play in the pathogenesis of the inflammatory arthritides that afflict man.

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